"THE NEUROENDOCRINE CONTROL OF BROODINESS"

by

Michael Charles MACNAMEE BSc.(hons.) MSc.

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ABSTRACT

The neuroendocrine control of broodiness was investigated in the bantam hen. The functional activity of hypothalamic monoaminergic neurones and the role of vasoactive intestinal polypeptide were considered in relation to the control of prolactin and luteinizing hormone (LH) secretion.

The functional activity of hypothalamic noradrenergic neurones, based on an index of turnover, did not change after the onset of incubation. However, the functional activity of hypothalamic adrenergic neurones was inversely correlated with plasma levels of LH and positively correlated with plasma levels of prolactin . This suggests that adrenaline-containing neurones may be, in part, responsible for the increase in plasma levels of prolactin and the decrease in plasma levels of LH at the onset of incubation. Similarly the functional activity of dopaminergic neurones in the anterior hypothalamus, but not the posterior hypothalamus, was inversely correlated with plasma LH and positively correlated with plasma prolactin.

Putative dopamine receptors were demonstrated in both the hypothalamus and the pituitary gland. Hypothalamic putative dopamine receptors were the same in laying and incubating birds. The density of putative dopamine receptors was significantly lower in pituitary glands from incubating birds than in those of laying birds. This suggests that any direct inhibitory influence of dopaminergic neurones in the posterior hypothalamus on prolactin secretion in incubating birds is reduced by 'down-regulation' of dopamine receptors in the anterior pituitary gland.

The distribution of 5-hydroxytryptamine (5HT) containing neurones in the brain-stem and hypothalamus was mapped using an immunohistochemical technique. Extensive 5HT fibres and terminals originating from cell bodies in the hind-brain and paraventricular organ were abundant throughout the hypothalamus with the highest densities occurring in the pre-optic area and basal hypothalamus. Putative 5HT receptors were demonstrated in the hypothalamus but were absent from the anterior pituitary gland. Within the anterior hypothalamus a unique population

of putative 5HT receptors was found only in the laying bird. This population disappeared during the onset of broodiness. The disappearance of this receptor population may be in part responsible for an increase in the functional activity of 5HT neurones in the anterior hypothalamus which was observed after the onset of incubation. The functional activity of 5HT containing neurones in the posterior hypothalamus did not change after the onset of incubation.

The possible role of hypothalamic 5HT-containing neurones in the stimulation of prolactin secretion was investigated by assessing the influence of various drugs known to alter 5HT function, on plasma levels of prolactin. Although the drug studies suggested a strong stimulatory influence of 5HT neurones on prolactin secretion when it was measured in a homologous chicken radioimmunoassay these findings were not confirmed when prolactin was measured in a heterologous radioimmunoassay. Separation of the proteins present in various *in vitro* incubations of pituitary glands and hypothalami by polyacrylamide gel electrophoresis demonstrated that the homologous radioimmunoassay cross-reacted with proteins of hypothalamic origin as well as prolactin released from the pituitary gland. It was concluded that, although 5HT-containing neurones may be involved in the stimulation of a prolactin releasing factor from the hypothalamus, their role in the neuroendocrine control of broodiness is limited.

In the hypothalamus a vasoactive intestinal polypeptide-like substance was located immunohistochemically in neurones in the basal hypothalamus which had an anatomical distribution consistent with a role in the control of anterior pituitary gland function. Vasoactive intestinal polypeptide stimulated selectively prolactin secretion when administerd *in vivo*, and stimulated prolactin secretion from the isolated pituitary gland. These observations suggest that endogenous hypothalamic vasoactive intestinal polypeptide is a specific prolactin-releasing factor in the bantam hen. It is also suggested that the stimulation of prolactin secretion associated with the hypothalamic monoamine neurotransmitters may be mediated in part by vasoactive intestinal polypeptide-containing neurones.

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DECLARATION

I declare herewith that I have completed this thesis without any further help than acknowledged and that I have not presented this thesis or any part of this thesis in fulfilment of another degree.

I N D E X

1.0 INTRODUCTION

1.1 BROODINESS IN THE CHICKEN: PHYSIOLOGICAL AND BEHAVIOURAL CORRELATES

In the chicken the periods of incubation and parental care , although physiologically distinct (Saeki and Tanabe, 1954), are collectively referred to as broodiness. Domestication and selective breeding for increased egg production have reduced the incidence of broodiness in commercial poultry. However, some heavy strains of laying hens, broiler breeders and turkey hens still retain a broody tendency, albeit reduced. The loss of egg production due to broodiness and the labour intensive nature of the current treatments used to remedy broodiness (Sharp, 1979) are considerable financial burdens on an otherwise extremely efficient industry. A better understanding of the neuroendocrine control of broodiness, as well as being of interest in itself, may alleviate some of this burden by providing the basis for rational remedial treatments for broodiness.

Several physiological and behavioural attributes distinguish the broody from the laying hen. Towards the onset of incubation, follicles in the ovary become atretic , egg production stops and the ovary and oviduct regress. Also, because of their dependence on gonadal steroids, the comb and wattles lose their bright red colour. In chickens a single large brood patch develops at the onset of incubation in response to the combined actions of prolactin and the gonadal steroids (Jones et al. 1970). The hen's behaviour also changes at this time. Continuous incubation is preceded by an increase in nesting at night which progressively extends into the end of the light period (Lea et al. 1981). As incubation gets underway hens begin to display a characteristically crouched posture, with the wings extended laterally, while on the nest and during infrequent feeding trips off the nest. Aggressive defence of the nest is also shown, accompanied by feather erection, pecking and a 'clucking' call. After the eggs hatch, defence of the chicks and foraging with the chicks are the predominant behaviours. As the chicks become independent the hen's ovary and oviduct gradually re-develop, the brood patch refeathers and laying resumes.

1.1.1 Prolactin and Broodiness

The first experimental evidence suggesting the involvement of prolactin in broodiness was reported by Riddle, Bates and Lahr in 1935. They found that "the full expression of broodiness" could be induced in laying hens by injections of a crude chicken prolactin preparation. Similar injections given to non-laying hens and laying hens of a non-broody strain, resulted in clucking and care of chicks but not incubation. Subsequent investigations demonstrated that homogenates of pituitary glands from broody hens induced a bigger response in the pigeon crop sac bioassay for prolactin than pituitary homogenates from laying hens (Burrows and Byerley, 1936). This shows that pituitary glands from broody hens contain more prolactin than pituitary glands from laying birds. It has also been demonstrated that the pituitary glands of hens brooding chicks contain less prolactin than those of incubating hens (Saeki and Tanabe, 1954 , 1955). Early cytological studies showed a change in the structure of both the caudal and cephalic lobes of the anterior pituitary glands of broody hens (Payne, 1942 , 1943; Yasudo, 1953). Later it was demonstrated that the caudal lobe of a pituitary gland from a broody hen contained as much, if not more prolactin than the cephalic lobe while little prolactin was found in the caudal lobe of pituitary glands from non-broody hens (Nakajo and Tanaka, 1956).

The early studies of Riddle and Lahr (1944) on ring doves suggested that, as in the chicken, prolactin is responsible for the initiation of incubation. However, later observations did not support this view since it was found that progesterone is more effective than prolactin in inducing incubatory behaviour in this species (Lehrman, 1958; Lehrman and Brody, 1961). Also, using the pigeon crop-sac radioreceptor assay to measure prolactin, Buntin and Forsyth (1979) found that pituitary levels of prolactin in ring doves do not rise until after the onset of incubation. Recent radioimmunoassay evidence confirmed this finding, demonstrating that plasma levels of prolactin do not increase until after incubation has began (Goldsmith et al. 1981). Despite the uncertainty about prolactin's involvement in the induction of incubation, its role in the maintenance of incubation and

the parental care of the young in the ring dove is generally accepted (Lehrman and Brody, 1964; Silver, 1978). Its specialised function in promoting the development of the crop sac and production of crop 'milk' in this species requires that prolactin secretion be delayed until near the hatch of the squabs. As this is not the case in chickens, findings based on experiments in the ring dove may be of questionable relevance to the study of the role of prolactin in broodiness in the bantam hen.

Although, in contradiction to the early investigations, a recent study found that a highly purified mammalian prolactin preparation failed to induce broodiness when administrated to chickens and turkeys (Opel and Proudman, 1980), the development of radioimmunoassays allowing the measurement of plasma levels of prolactin, has firmly established the involvement of prolactin in broodiness in poultry. Using a homologous chicken radioimmunoassay (Scanes et al. 1976), Sharp et al. (1979) observed a small but significant rise in plasma levels of prolactin during incubation in bantam hens. A larger rise was observed in White Rock hens using an improved homologous assay (Bedrak et al. 1981). Lea et al. (1981) demonstrated a large rise in plasma levels of prolactin around the onset of incubation in bantam hens. This rise was also observed using a heterologous prolactin radioimmunoassay based on an antiserum to human prolactin and ovine prolactin label developed in 1978 for birds by McNeilly et al. (Lea et al. 1981; Lea, 1982). Although no change in plasma levels of prolactin were apparent in the broody turkey when measured in the homologous chicken assay (Harvey et al. 1981) or the heterologous assay (Etches et al. 1979), large increases in plasma levels of prolactin in broody turkeys have been measured (Burke and Dennison, 1980; Lea and Sharp, 1982) using a homologous turkey radioimmunoassay developed by Burke and Papkoff (1980). The finding in the bantam hen, that plasma levels of prolactin begin to rise prior to the onset of incubation suggests that prolactin may play a role in the initiation of this behaviour (Lea et al. 1981). In the bantam hen, in addition to a possible involvement in the induction of incubation, prolactin is also involved in the maintenance of this behaviour since injections of ovine prolactin maintain broody behaviour in birds deprived of their nests (Sharp,P.J. and Pedersen,H.C. unpublished observations).

1.1.2 Luteinizing Hormone, Gonadal Steroids and Broodiness

In bantam hens plasma levels of luteinizing hormone (LH) and progesterone are high when the birds are laying but fall quickly at the onset of incubation (Sharp et al. 1979). The fall in plasma levels of LH and progesterone is preceded by an increase in plasma levels of prolactin at the onset of incubation (Lea et al. 1981). The inverse relationship between prolactin and LH may be due to the ability of prolactin to inhibit gonadotrophin secretion by inhibiting the release of luteinizing hormone releasing hormone (Lea and Sharp, 1982). In the turkey (Cogger and Burke. 1976; Cogger et al. 1979; Burke and Dennison. 1980) and chicken (Bedrak et al. 1981) plasma levels of LH, progesterone and oestradiol decline before the onset of incubation. Plasma levels of LH are rapidly restored following the removal of eggs and/or chicks from broody birds of several species including bantam hens (Sharp et al. 1979) and turkeys (Lea and Sharp 1982; Lea, 1982). This increase in LH secretion may be a consequence of a decrease in prolactin release since, as observed in turkeys, nest deprivation results in a rapid fall in plasma levels of prolactin (El Halawani et al. 1983).

From this evidence and assuming the appropriate environmental conditions exist, the induction and maintenance of broodiness involves a decrease in gonadotrophic activity, and consequently gonadal steroid secretion, and an increase in prolactin secretion.

1.2 THE CONTROL OF THE PROLACTIN SECRETION.

The ability of anterior pituitary gland extract to induce lactation in pseudo-pregnant rabbits was first demonstrated in the 1920's (Stricker and Grueter ,1928). Similar findings coupled with the ability of anterior pituitary gland extract to induce the formation of crop 'milk' in pigeons led Riddle et al.(1933) to name this newly discovered hormone prolactin. In the pigeon, acidophilic cells in the anterior pituitary were identified as responsible for prolactin production

(Schooly and Riddle, 1938), and in an early study structurally comparable acidophilic cells were also found in the fowl and mammalian anterior pituitary gland (Rahn, 1939).

The chemical composition of avian prolactin, first isolated from chicken (Scanes et al. 1975) and subsequently, turkey pituitary glands (Burke and Papkoff, 1980; Proudman and Corcoran, 1981), is closely related to that of ovine prolactin and is distinct from avian growth hormone.

Intensive study has shown that prolactin has many different actions in many species (Nicoll, 1974; Swearingen and Nicoll, 1974). These actions affect aspects of reproduction, behaviour, osmoregulation, growth and metabolism (de Vlaming, 1974). Much is known of the control of prolactin secretion in mammals (reviews : Neill, 1974; Tindall, 1974; MacLeod, 1976; Meites, 1977; Boyd and Reichlin, 1978; Enjalbert et al. 1978; Lancranjan and Friesen, 1975; MacLeod and Scapagnini, 1980) while less is known about its control in birds (Harvey et al. 1982; Chadwick and Hall, 1983) The purpose of this section is to discuss briefly the control of prolactin secretion in mammals and birds.

1.2.1 The Control of Prolactin Secretion in Mammals.

In mammals, prolactin secretion is under the predominantly inhibitory influence of the hypothalamus. The first indication of such an inhibition was the report that rat anterior pituitary glands grafted away from the hypothalamus retained their luteotrophic actions (Desclin, 1950) and that pituitary transplants secreted prolactin autonomously (Everett, 1954). Later research showed that hypothalamic factors could inhibit prolactin release in vitro (Pasteels, 1962; Ratner and Meites, 1964) and that they were present in hypophysial portal blood in vivo (Kamberi et al. 1971). There is now general agreement that the catecholamine, dopamine, is the major physiological prolactin inhibiting factor in mammals(see Shaar and Clemens, 1974). Dopamine released from the terminals of tubero-infundibular neurones of the median eminence (Weiner and Ganong, 1978) into the hypophysial portal blood inhibits the secretion of prolactin by the activation of

specific dopamine receptors located on the prolactin producing cells of the pituitary gland (Brown et al. 1976). There is evidence, however, that dopamine is not the sole prolactin inhibiting factor in the mammalian hypothalamus (Kordon and Enjalbert, 1980). Attempts to isolate peptide prolactin inhibiting factors have not yet been successful.

The existence of a prolactin-releasing factor in the mammalian hypothalamus has been suggested (Nicoll et al. 1970; Valverde et al. 1972). Thyrotropin-releasing hormone (TRH) stimulates prolactin release from cultured rat pituitary cells (Tashjian et al. 1971) and is considered a possible releasing factor. However, prolactin release is not impaired after passive immunisation with anti-TRH serum (Harris et al. 1978). Several other peptides including, the opioid peptides (Rivier et al. 1977; Wardlaw et al. 1980), bombesin, ranatensin, and litorin (Rivier et al. 1978), arginine vasotocin (Johnson, 1978), neurotensin and substance P (Rivier et al. 1977) and vasoactive intestinal polypeptide (Ruberg et al. 1978) have been shown to stimulate prolactin secretion. However, only vasoactive intestinal polypeptide (VIP) stimulates prolactin secretion by a direct action on the pituitary gland. The dose-response relationship between VIP and prolactin secretion in vitro (Clemens et al. 1980), its localisation in the hypothalamus (Samson et al. 1978) and its occurrence in higher concentrations in hypophysial portal blood than in the peripheral circulation (Said et al. 1978; Said and Porter, 1979) suggest that VIP is a physiological prolactin releasing factor .

In mammals, prolactin exerts a negative feedback action on its own secretion. This feedback occurs at the level of both the pituitary gland (MacLeod and Abad, 1968; Nicoll, 1971; Herbert et al. 1979) and the hypothalamus (Chen et al. 1967; Meites and Clemens, 1972). Prolactin selectively increases the release of dopamine from the terminals of the tubero-infundibular dopaminergic neurones, thus inhibiting its own release (Hockfelt and Fuxe, 1972; Annunziato and Moor, 1978; Gudelsky and Porter, 1980). Conversely, oestrogen stimulates prolactin secretion in mammals by making the pituitary gland

less sensitive to dopaminergic inhibition (Raymond et al. 1978) and by exerting anti-dopaminergic effects in the hypothalamus (Euvard et al. 1979).

In addition to dopamine, other hypothalamic monoamines are also involved in the control of prolactin secretion. Neurones in the mammalian hypothalamus containing 5-hydroxytryptamine appear to have a stimulatory role in the regulation of prolactin release in vivo (Lu and Meites, 1973), and probably stimulate the release of a prolactin-releasing factor (Clemens et al. 1975). The possible role of noradrenaline in the control of prolactin secretion is less clear, since inhibitory and stimulatory effects have been reported (Clemens and Shaar, 1980). There is agreement, however, that noradrenaline does not act directly on the pituitary gland and probably alters the balance of inhibitory and stimulatory influences on prolactin secretion by a hypothalamic mechanism.

Studies involving the electrical stimulation (Everett and Quinn, 1966; Wuttke and Meites, 1972; Tindall and Knaggs, 1969; Peters and Gala, 1975) and ablation of localised areas of the hypothalamus (Wolinska et al, 1977) support the view that the control of prolactin secretion in mammals involves both inhibitory and stimulatory mechanisms (Domanski and Polkowska, 1973). Inhibitory control appears to be mediated by the lateral part of the medio-basal hypothalamus while stimulatory control seems to be predominantly a function of the supra-chiasmatic region.

1.2.2. The Control of Prolactin Secretion in Birds

The control of prolactin secretion in birds differs fundamentally from that in mammals in as much as the avian hypothalamus exerts a predominantly stimulatory influence over the pituitary gland. Supporting evidence for this comes from the findings that the pituitary glands of pigeons (Tixier-Vidal and Gourdji, 1972) and chickens (Bolton et al. 1974) do not maintain a large output of prolactin when incubated in vitro. Also auto-transplantation and homo-transplantation of the pituitary glands in chickens (Ma and Nalbandov, 1963) and pigeons (Bayle, 1969; Bayle and Assenmacher, 1965) showed that prolactin

secretion is not maintained when the influence of the hypothalamus is removed. However, chicken pituitary glands do secrete small quantities of prolactin when incubated in vitro (Harvey et al. 1982), suggesting a small degree of autonomous secretion. The Peking Duck would seem to be an exception amongst the species so far studied in that prolactin secretion is maintained at a relatively high level in the medium of cultures of pituitary cells from this species (Gourdji and Tixier-Vidal, 1966; Tixier-Vidal and Gourdji, 1970).

Further evidence of a stimulatory role for the hypothalamus in the control of prolactin secretion comes from the study of the effects of hypothalamic extract on prolactin release. In vitro incubation of pigeon (Kargt and Meites, 1965), duck (Gourdji and Tixier-Vidal, 1966), tri-coloured blackbird (Nicoll, 1965), turkey(Chen et al. 1968), quail (Tixier-Vidal and Gourdji, 1972) and chicken (Hall et al. 1975) pituitary glands with hypothalamic extract results in a significant increase in prolactin secretion. In vivo administration of hypothalamic extract to pigeons stimulates prolactin secretion sufficiently to cause crop sac development (Knight and Chadwick, 1975). Also, small amounts of hypothalamic extract greatly elevate plasma levels of prolactin within minutes of injection in both conscious and anaesthetised chickens (Bolton et al. 1976; Chadwick et al. 1978; Harvey et al. 1979). The rise in plasma levels of prolactin is dependent on the dose of hypothalamic extract administered (Harvey et al. 1979).

The ability of rat hypothalamic extract to inhibit the rise in plasma levels of prolactin following the administration of chicken hypothalamic extract to chickens (Bolton et al. 1976) raises the possibility that prolactin secretion in the chicken may be, in part, under inhibitory hypothalamic control. Since dopamine is a prolactin-inhibiting factor in mammals it could also serve this function in birds. In support of this view dopamine inhibits the basal release (Harvey et al. 1982) and the stimulated release (Hall et al. 1975) of prolactin from chicken pituitary glands incubated in vitro . Although dopamine is present in the chicken median eminence and hypothalamus (Talbot et al. 1984), there is no evidence of a dopaminergic system equivalent to the tubero-infundibular pathway

described in mammals (Ikeda and Gotoh, 1971). However, the analogue has been drawn between the avian paraventricular organ and the mammalian tubero-infundibular system (Okshe ,1983).

Stimulation of prolactin release from the isolated chicken pituitary gland by 5-hydroxytryptamine has been reported (Border and Chadwick, 1977), but this effect is much enhanced in pituitary-hypothalamus co-incubations (Harvey et al. 1982). These findings suggest that 5-hydroxytryptamine acts predominantly through the hypothalamus to release prolactin. In vivo, administration of 5-hydroxytryptamine stimulates prolactin secretion in the pigeon (Nistico et al. 1980). Further , in cockerels, 5-hydroxytryptamine receptor agonists cause an increase, while antagonists cause a decrease in plasma levels of prolactin (Rabii et al. 1981). Noradrenaline also stimulates prolactin release from the isolated chicken pituitary gland, and enhances prolactin release in pituitary/hypothalamus co-incubations (Harvey et al. 1982). Monoamine neurotransmitters would thus seem to exert a predominantly stimulatory effect on prolactin secretion. However, when chicken hypothalamic extract is treated with alumina to absorb monoamines it loses only about 50% of its ability either to release prolactin from the chicken pituitary gland in vitro, or to elevate plasma levels of prolactin in chickens in vivo (Border, 1979). These findings imply the existence of a non-monoaminergic prolactin releasing factor in the chicken hypothalamus.

As thyrotrophin-releasing hormone (TRH) is a potent prolactin releasing factor in mammals, its possible physiological significance in the control of prolactin secretion in birds has been studied. Initial investigations demonstrated that TRH stimulates prolactin secretion from chicken and pigeon pituitary glands, incubated in vitro (Bolton et al. 1974; Chadwick and Hall, 1975; Hall et al. 1975). Hall et al. (1975) found that TRH stimulated both prolactin and growth hormone release from pigeon pituitary glands and that this effect is inhibited by dopamine. Using the homologous chicken radioimmunoassay, the stimulatory effect of TRH on prolactin release from isolated chicken pituitary glands was confirmed, but was not apparent in vivo (Harvey et al. 1978a). Also, ~~the administration of anti-TRH serum to pigeons~~

~~resulted in a proliferation of the crop sac mucosa (Shani et al. 1980)~~, ~~while~~ the administration of anti-TRH serum to incubating bantam hens had no effect on plasma levels of prolactin measured by radioimmunoassay(Macnamee,M.C.and Sharp,P.J. unpublished observations). Further, electrical stimulation of the pre-optic region of the pigeon hypothalamus results in a stimulation of crop sac growth which is not mimicked by injections of TRH (Kanematsu, 1980). The reconciliation of these apparently contradictory findings awaits further investigation. The observation that the concentration of TRH estimated in chicken hypothalamus is too low to account for all the prolactin-releasing activity of hypothalamic extract, suggests that TRH is not the sole prolactin releasing factor in birds (Jackson and Reichlin, 1974).

Another potent prolactin-releasing factor in mammals is vasoactive intestinal polypeptide (VIP). Four amino-acid substitutions differentiate mammalian VIP from VIP isolated from chicken gut (Nilsson, 1974,1975). Although chicken VIP has been demonstrated to have biological actions on the gastro-intestinal tract (Dimaline and Dockray, 1979) and a VIP-like substance has been immunohistochemically localised in the quail hypothalamus (Yamada and Mikami, 1982), there has been no investigation of a possible physiological role for VIP in the regulation of prolactin secretion in birds.

The effects of oestrogen on prolactin release in birds ~~is~~ are similar to those in mammals. Oestrogen treatment stimulates prolactin release from the isolated chicken pituitary gland, and enhances prolactin release in pituitary/hypothalamus co-incubations (Hall and Chadwick, 1978).It also elevates plasma levels of prolactin in non-laying, but not in laying turkey hens (McNeilly et al. 1978).

Although there are some general similarities in the ways in which prolactin secretion is influenced in birds, there seem to be important species and strain differences. For example the rise in plasma prolactin associated with oestrogen treatment in turkeys (McNeilly et al. 1978), does not occur in the bantam hens (Sharp,P.J. Sterling,R. and Macnamee,M.C., unpublished observations). Further, turkeys and chickens seem to respond differently to photostimulation in terms of

prolactin secretion (Lea, 1982), and the pre-ovulatory surge of LH is accompanied by a decrease in plasma levels of prolactin in the chicken but not in the turkey(Opel et al. 1983).

1.3 THE CONTROL OF LUTEINIZING HORMONE SECRETION IN BIRDS.

As was previously suggested (section 1.1), an inverse relationship exists between prolactin and luteinizing hormone (LH) secretion. This relationship is apparent in the bantam hen at the onset of incubation (Lea et al. 1981)and during nest deprivation (see section 4.1.1.), and also occurs during the reproductive cycle in mallards (Goldsmith and Williams, 1980). It was also observed in the hen during the ovulatory cycle where the pre-ovulatory LH surge is associated with a rapid fall in plasma levels of prolactin (Scanes et al, 1977). In mammals, there is a similar reciprocal relationship between LH and prolactin. Thus, during suckling, prolactin secretion rises and LH secretion falls. These hormonal changes are correlated with an increase in the turnover of 5-hydroxytryptamine in the hypothalamus (Meites and Sonntag, 1981). The reciprocal relationship between prolactin and LH in birds suggests an interaction between the mechanisms controlling their secretion. The purpose of this section is to summarise briefly the factors which influence the secretion of LH in the chicken.

The central role of the avian hypothalamus in the regulation of LH secretion is well established (Sharp, 1983). The distribution of the decapeptide luteinizing-hormone-releasing-hormone (LHRH) within the chicken hypothalamus has been described (Jozsa and Mess, 1982; Sterling and Sharp, 1982) and two forms of specific chicken LHRH have been isolated (King and Miller, 1982; Miyamoto et al. 1982 , 1983; Miller and King, 1983). The secretion of LH is primarily controlled by the activity of the LHRH system, which is influenced by other neural inputs, which in turn are influenced by external stimuli and stimuli resulting from the activity of hypothalamo-pituitary-gonadal system.

Three components involved in the control of LH secretion have been identified; a direct steroid-independent 'hypothalamic drive', a steroid negative feedback and a steroid positive feedback system (Sharp, 1983).

In the mature bird the steroid-independent mechanism is based on complex interactions of environmental factors (Murton and Westwood, 1977). Changes in its activity directly influence the function of the LHRH system and may be responsible for the initiation and termination of breeding. At all stages of the breeding cycle the control of LH secretion is also influenced by steroid feedback mechanisms.

Increases in LH secretion have been reported following gonadectomy in many avian species including chickens (Sharp, 1975; Wilson and Sharp, 1976), ~~which is~~ consistent with a negative influence of gonadal steroids on LH secretion. Also numerous studies have shown that the administration of gonadal steroids inhibits gonadotrophin secretion (Sharp, 1980). Wilson and Sharp (1976) found that oestrogen, and to a lesser extent, progesterone administration depressed LH release in ovariectomized hens and that a greater suppression of LH secretion is achieved by their combined administration rather than the sum of their individual effects. The pituitary gland is the major site for this inhibitory feedback. As the ovary develops, and plasma levels of the ovarian steroids increase, the response of the pituitary to LHRH decreases significantly (Wilson and Sharp, 1975). Also oestradiol can directly inhibit LH release and antagonize the action of LHRH on pituitary cells in culture (Bonney and Cunningham, 1977; Luck and Scanes, 1980).

The positive feedback influence of the gonadal steroids is concerned with the regulation of the preovulatory release of LH. In a cascade mechanism, an increase in circulating LH stimulates the secretion of ovarian steroids which in turn stimulates further LH release (Sharp, 1980). Progesterone is the most likely positive feedback steroid in the hen (see Sharp, 1983). However, progesterone injections fail to trigger LH release in ovariectomised hens unless the birds are pre-treated for several days with both oestrogen and progesterone (Wilson and Sharp,

1976). This observation shows that the positive feedback stimulation of LH secretion involves both oestrogen and progesterone during the "priming" phase, and only after the positive feedback mechanism is primed can an increase in circulating levels of progesterone trigger the preovulatory surge of LH. The priming effect of oestrogen is thought to be due to its stimulatory effect on the formation of progesterone receptors (Sharp, 1983). Both cytoplasmic and nuclear progesterone receptors occur in the hypothalamus and pituitary gland of the hen (Kawashima et al. 1978 ,1979a). The concentrations of these cytoplasmic receptors change during the ovulatory cycle (Kawashima et al. 1979b) in such a way as to suggest their involvement in the effects of progesterone in the preovulatory release of LH. However ,it has been recently demonstrated that LHRH cells in the chicken hypothalamus do not contain nuclear progesterone receptor material (Sterling et al. 1984). This, together with the rapidity with which progesterone can stimulate LH secretion (Wilson and Sharp, 1972), suggests that progesterone acts on the LHRH system directly, or through an effect on other neurones of as yet unidentified type, which contain nuclear progesterone receptor.

The current understanding of the role of monoamines in the control of gonadotrophin secretion is based on correlations between hypothalamic monoamine content and changes in reproductive activity, and the effects of pharmacological manipulation on LH release. Pharmacological treatments in quail have demonstrated that noradrenaline stimulates, while both dopamine and 5-hydroxytryptamine inhibit, photoperiodically-induced testis growth (El Halawani et al. 1975 ,1978). Evidence that these transmitters are involved in the control of LH secretion comes from studies involving the measurement of plasma LH levels rather than testicular growth (El Halawani et al. 1980a). Pharmacological studies on juvenile hens confirm the stimulatory effect of noradrenaline and the inhibitory effect of both dopamine and 5-hydroxytryptamine on LH secretion(Buonomo et al. 1981; Buonomo and Scanes, 1983). In sum these observations suggest the involvement of noradrenergic neurones in the stimulation, and dopaminergic and 5HT neurones in the inhibition, of the non-steroid dependent control of LH secretion.

In the turkey, the post-castration rise in plasma levels of LH is accompanied by an increase in the concentration and turnover of noradrenaline in the hypothalamus (El Halawani et al. 1980b) In the chicken, the rise in plasma levels of LH after ovariectomy is accompanied by increased concentrations of dopamine and noradrenaline in the medio-basal hypothalamus and increased concentrations of noradrenaline and adrenaline in the anterior hypothalamus (Knight et al. 1981). These findings suggest the inhibitory feedback of the gonadal steroids may be due to their effect on catecholaminergic neurones in the hypothalamus.

The progesterone-induced release of LH in the hen is also accompanied by changes in the concentrations of monoamines in the hypothalamus, with both dopamine and noradrenaline concentrations increased in the pre-optic region (Knight et al. 1982). The spontaneous preovulatory surge of LH in the hen is associated with a decrease in the turnover of both 5-hydroxytryptamine and dopamine in the hypothalamus (Talbot et al. 1984). These findings suggest the involvement of both catecholaminergic and 5HT neurones in the hypothalamus in the positive feedback action of the gonadal steroids on LH release.

In the reproductive cycle of the bantam hen egg production terminates with the onset of incubation. The finding that injection of synthetic mammalian LHRH increased plasma levels of LH in incubating bantams (Sharp and Lea, 1981), suggests that LH secretion may be suppressed because of a reduction in the secretion of endogenous LHRH. The evidence presented above suggests that such a reduction may be brought about by changes in the activity of hypothalamic monoaminergic neurones.

1.4. THE BIOCHEMISTRY OF MONOAMINE NEUROTRANSMITTER SYNTHESIS AND METABOLISM

Monoaminergic neurotransmission in the avian brain has been implicated in the control of gonadotrophin and prolactin secretion in birds (see section 1.2 and 1.3). The purpose of this section is to review briefly the current understanding of the biochemistry of monoamine neurotransmitter synthesis, storage and metabolism, which forms the basis of the estimation of hypothalamic monoamine functional activity in the present work (section 4).

1.4.1. The Catecholamines

Knowledge of the cellular sites and mechanisms of synthesis, storage and release of the catecholamine neurotransmitters ~~are~~ is derived from studies of adrenergically innervated organs and adrenal medullary tissue. The pathway of catecholamine synthesis (Figure 1) was originally suggested by Blaschko (1939) and Holtz (1939), but the complete pattern of anabolism was not demonstrated until Gurin and Delluva (1947) gave radio-labelled phenylalanine to rats and recovered radio-labelled adrenaline from the adrenal glands.

Catecholamine synthesis occurs in all parts of the adrenergic neurone, the three principal enzymes involved in noradrenaline synthesis; tyrosine hydroxylase, DOPA-decarboxylase, and dopamine-B-hydroxylase, being present in the cell body, and in both the non-terminal and terminal sections of axons (Goodall and Kirshner, 1958; Goldstein et al. 1972). Tyrosine hydroxylation and DOPA-decarboxylation occur in the axoplasm while B-hydroxylation of dopamine occurs solely in the adrenergic storage organelles (Weiner 1970; von Euler, 1972). The enzyme catalysing the conversion of noradrenaline to adrenaline, phenylethanolamine-N-methyltransferase (PNMT) although predominantly located in the adrenal glands, is also present in the axoplasm of some adrenergic neurones in the brain (Axelrod, 1966; Goldstein et al. 1972).

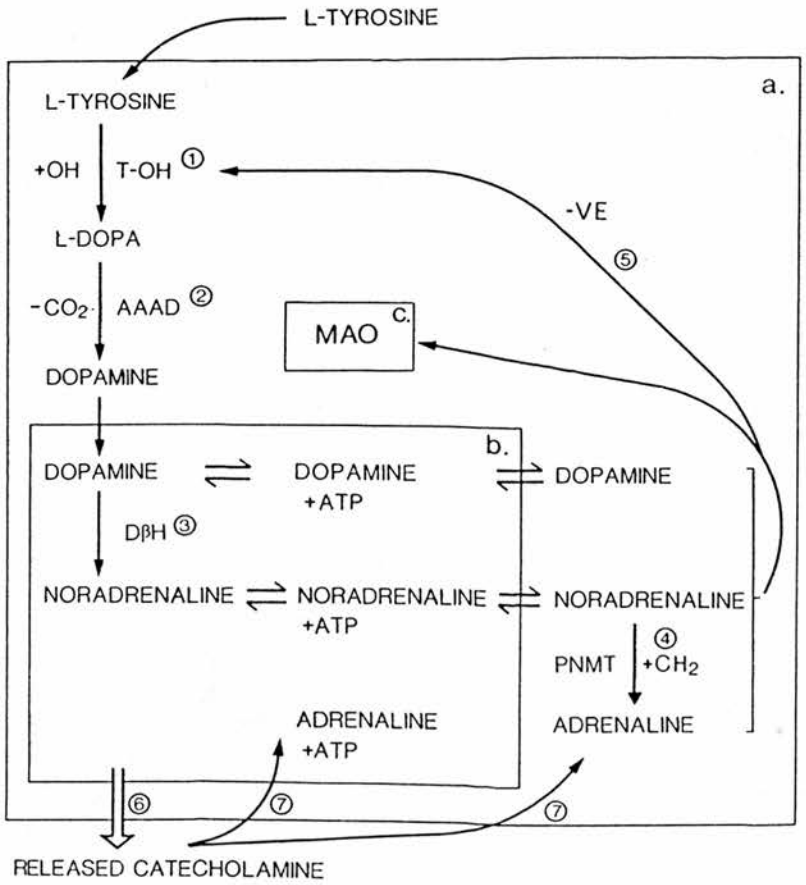


Figure 1: The biosynthesis of catecholamines in adrenergic neurones. Within the adrenergic neurone (a) l-tyrosine is converted by tyrosine hydroxylase (1) to l-DOPA, which is converted by aromatic amino acid decarboxylase (2) to dopamine. Within the storage granule (b), dopamine may be converted to noradrenaline by the action of dopamine-β-hydroxylase (3). Adrenaline can be produced by the action of phenethanolamine-N-methyl transferase (4) on cytoplasmic noradrenaline. Catecholamines released by exocytosis following nerve stimulation (6) are taken up by the neurone (7) for restorage or metabolism. Most intraneuronal metabolism is carried out by the mitochondrial enzyme monoamine oxidase (c). The enzyme T-OH is subject to end product inhibition (5).

The hydroxylation of tyrosine is the rate-limiting step in the synthesis of the catecholamines (Spector et al. 1963; Levitt et al. 1965; Udenfriend, 1966). The activity of tyrosine hydroxylase in nerve terminals is subject to end product inhibition (Nagutsu et al. 1964; Spector et al. 1967; Weiner, 1970), with noradrenaline competing for its pteridine cofactor, providing a fast-feedback regulation of catecholamine synthesis. Long-term changes in the rate of catecholamine synthesis are achieved by regulation of the synthesis of tyrosine-hydroxylase, which is dependent on neuronal activity (Axelrod et al. 1970; Thonen, 1970; Thonen et al. 1970 , 1971; Bhatnagar and Moore, 1972). The metabolic degradation of tyrosine hydroxylase is slow, with a half-life of at least 8 days (Meuller et al. 1969). So, by short-term end product inhibition and by control of tyrosine hydroxylase synthesis, the synthesis of the catecholamine neurotransmitters can be adapted to changes in the patterns of neural activity.

The biological effects of catecholamines released from the adrenergic neurone are terminated largely by reuptake to axons and other cells surrounding the synapse (Iversen, 1971). Once inside the cell, catecholamines which have been re-uptaken can be stored or metabolised. The continuous degradation of catecholamine neurotransmitters occurs in a wide variety of tissues and is performed by the combined actions of monoamine oxidase and catechol-O-methyl transferase (Axelrod, 1966; Kopin, 1972; Sharman, 1972). Monoamine oxidase is a generic term for a group of enzymes which catalyse the oxidative deamination of monoamines (see Blaschko, 1972) which are located within the double outer membrane of mitochondria (Snyder et al. 1965; Tipton, 1967; Jarrott, 1971a). At least two, functionally different, monoamine oxidase enzymes have been identified, both of which occur in rat brain (Youdini et al. 1983). Catechol-O-methyltransferase is widely distributed in many tissues. However, only a relatively small amount is found in the cytoplasm of adrenergic neurones (Jarrot, 1971b; Klingman and Klingman, 1972 a and b). In normal circumstances monoamine oxidase and catechol-O-methyl transferase act in concert each providing substrate for the other (see figure 2).

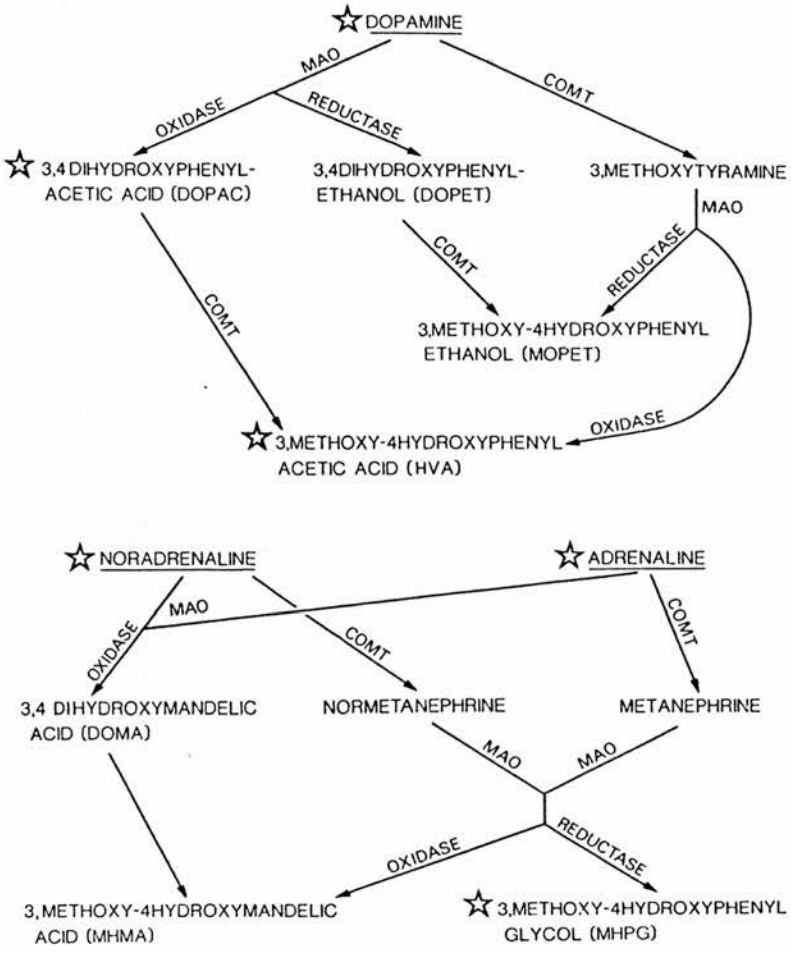


Figure 2: The metabolic pathways of the catecholamine neurotransmitters. All catecholamines are liable to metabolism by both monoamine oxidase (MAO) and catechol-O-methyl transferase (COMT). Substance detectable by electrochemical detection after HPLC (see section 2.4) are marked by *.

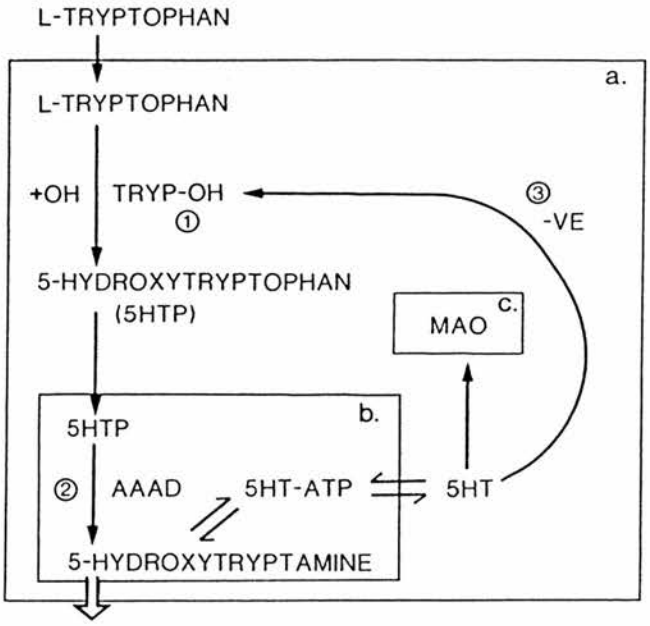
1.4.2. 5-Hydroxytryptamine

5-hydroxytryptamine was identified in the mammalian brain in 1953 (Twarzog and Page, 1953; Amin et al. 1954) and in the avian brain in 1956 (Correale, 1956; Bogdanski et al. 1963). Since then compelling evidence has accumulated suggesting that 5-hydroxytryptamine serves as the neurotransmitter substance of a distinct class of neurones (Chase and Murphy, 1973). The synthetic pathway for 5-hydroxytryptamine from dietary L-tryptophan has been demonstrated (Udenfriend, 1959) and is shown in figure 3.A. The enzymes concerned are tryptophan hydroxylase and dopamine-B-decarboxylase (more correctly termed aromatic amino acid decarboxylase). The hydroxylation of tryptophan takes place in the cytoplasm and the decarboxylation of 5-hydroxytryptophan is confined to the storage organelles (Bogdanski et al. 1957). The rate limiting step in this pathway is the hydroxylation of L-tryptophan and the enzyme tryptophan hydroxylase is subject to end-product inhibition. The factors governing its synthesis are similar to those regulating the synthesis of tyrosine hydroxylase in the adrenergic neurone (Macon et al. 1971; Carlsson and Lindqvist, 1972; Glowinski et al. 1972). The biological effects of released neuronal 5-hydroxytryptamine are terminated by re-uptake into the pre-synaptic neurone and other cells around the synapse where it can be metabolised only by the mitochondrial enzyme monoamine oxidase. Unlike the catecholamine neurotransmitters, 5-hydroxytryptamine is not a substrate for catechol-O-methyl transferase. The products of monoamine oxidase action on 5-hydroxytryptamine are shown in figure 3.B.

1.4.3. Functional Activity of Monoaminergic Neurones

The rate of monoamine neurotransmitter synthesis is dependent on the rate of hydroxylation of tyrosine for synthesis of the catecholamines and tryptophan for the synthesis of 5-hydroxytryptamine. In the short-term, the rate of monoamine neurotransmitter synthesis is regulated by end-product inhibition of tyrosine and tryptophan hydroxylase, which maintains the amount of transmitter in the neurone at a constant level. Longer-term adaptive changes in the rate of transmitter synthesis are governed by factors influencing the synthesis

a. Synthesis + storage of 5HT



b. Metabolism of 5HT by Monoamine Oxidase (MAO)

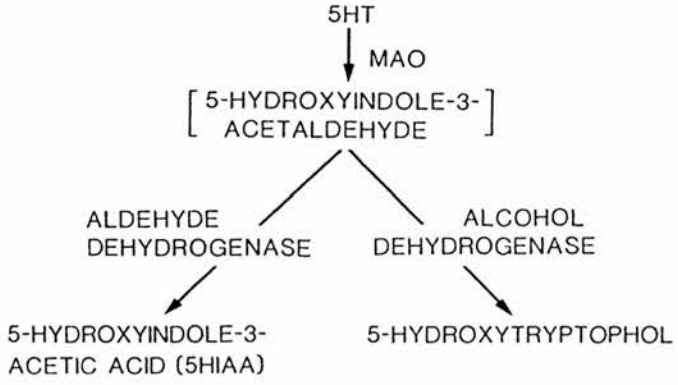


Figure 3: A; the synthesis of 5-hydroxytryptamine (5HT). Dietry l-tryptophan, once in the tryptaminergic neurone (a), is converted by tryptophan hydroxylase (1) to 5-hydroxytryptophan (5HTP). In the storage granule (b), 5HTP is converted to 5HT by aromatic amino acid decarboxylase(2). Released 5HT is reuptaken to the neurone where it can be metabolised by monoamine oxidase (3).
B; the metabolic pathway of 5HT. Monoamine oxidase (MAO) acts on 5HT to produce the unstable intermediary 5-hydroxyindole-3-acetaldehyde which is rapidly dehydrogenated to 5HIAA. 5HT, 5HTP and 5HIAA were measured by electrochemical detection after HPLC.

of these enzymes. The mechanism of changes in the rate of synthesis of the hydroxylases is activated by changes in neuronal activity (Burnstock and Costa, 1975; Hamon et al. 1977). The rate of synthesis of the monoamine neurotransmitter therefore reflects the functional activity of the monoaminergic neurone.

Several methods have been developed to estimate the turnover of the catecholamines in mammals. These include, a) the rate of disappearance of a radio-labelled amine after administration of the amine itself (Brodie et al. 1966; Costa and Neff, 1966), or of a radiolabelled precursor (Udenfriend and Zallman-Nirenberg, 1963; Burack and Draskoczy, 1964); b)the rate of disappearance of endogenous amines after synthesis inhibition (Brodie et al. 1966; Costa and Neff, 1966; Anden et al. 1969; Neff et al. 1969); c) the accumulation of radio-labelled amines after administration of labelled amino acids (Gordon et al. 1966; Neff et al. 1969); and d) the ratio of catecholamine metabolites formed (Carlsson and Lindqvist, 1963; Sharman, 1969). In the turkey, both the decrease in concentration of hypothalamic monoamines after blockade of synthesis (El Halawani et al. 1980b) and the accumulation of monoamines after inhibition of monoamine oxidase (El Halawani and Burke, 1976) have been used to estimate monoamine turnover rates.

Most of these methods involve pharmacological intervention in the normal functioning of the neurone and may therefore activate the adaptive mechanisms controlling monoamine synthesis discussed earlier (Malmfors, 1965; Presson and Waldeck, 1970). However, this does not limit their usefulness in comparative studies as the possible activation of such mechanisms would occur in all situations.

1.5. NEUROTRANSMITTER RECEPTORS

A chemical link in neurotransmission was first suggested at the turn of the century, when it was noted that the effects of injections of extracts of the adrenal glands bore a close resemblance to the stimulation of sympathetic nerves (Lewandowsky, 1898; Langley, 1901). The first real proof of the chemical mediation of nervous impulses was

supplied by Loewi (1921), who demonstrated that the perfusate of an isolated frog heart during vagal stimulation contained a substance which could slow the beat of another, non-stimulated, frog heart. Since then, overwhelming evidence supporting the theory of neurohumoral transmission has accumulated (Koelle, 1975). The theory of neurohumoral transmission holds that nerve impulses elicit responses in the innervated structure through the liberation of specific chemical substances. This involves the combination of the released chemicals with specific receptor sites, which are coupled to the biological machinery which can create a biochemical change in the target cell upon activation. The purpose of this section is to outline the development of receptor theory and the current understanding of receptor regulation.

1.5.1. Receptor Theory - Historical Development

Modern receptor theory originated from observations made at the turn of the century on the mode of action of drugs . Early speculation that the physiological actions of drugs were due to a chemical reaction between the drug and some constituent of the cell was supported by the work of Langley (1878) on the antagonism between atropine and pilocarpine on muscle contraction. Some years later, Erlich (circa 1897) suggested the existence of chemical 'side-chains' on cells to explain the binding of toxins. He distinguished between the toxophore group of toxins, which gave it its toxic properties, and the haptophore group which chemically bonded to 'side-chains' on the cell allowing the toxophore group to exert its action (see Himmelweit, 1956-1960). After demonstrating the direct action of nicotine on striated muscle, and that even in the absence of a nerve supply its actions could be antagonised by curare, Langley (1905) postulated that both drugs compete for a single 'receptive substance' on the muscle cell. Langley (1905) also suggested that the normal function of this 'receptive substance' was to transmit a stimulus from the nerve to the muscle.

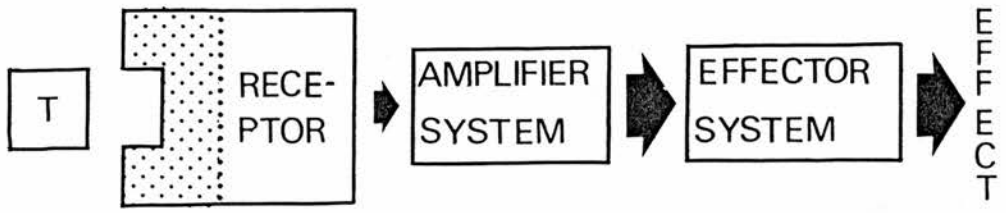
The impact of the receptor theory developed by Ehrlich and Langley was very limited in their lifetimes. The interaction of drugs and receptors did not receive any sort of quantitative expression until the work of

Clark and Gaddum in the 1920's and 1930's (Clark, 1926 a ,b; Gaddum, 1926 ,1937). Clark's work on the effects of atropine and acetylcholine at the neuromuscular junction led to the realisation that receptors were present in very small numbers and were most likely located on the cell membrane. Subsequent work using muscarine, atropine and nicotine, suggested the existence of two acetylcholine receptors, the so called 'muscarinic' and 'nicotinic' receptors. Similarly, study of the differential effects of adrenaline-like substances on the cardio-vascular system led Alquist (1948) to postulate the existence of two types of adrenoceptor, the alpha and beta receptors. Although the idea that one substance could produce opposite effects depending on the type of receptor on which it acted, met with much resistance at the time (see Alquist, 1973), it has subsequently proven to be of great value in explanation and quantification of the effects of sympathomimetic substances. The discovery of receptor sub-types, often with discrete localisation and mediating contrasting effects, led to the study of structure-activity relationships and the discovery of many specific agonists and antagonists for various receptors.

1.5.2. Current Models of Receptor Activation

The availability of specific receptor ligands and the advent of direct receptor measurement (Pert and Snyder, 1973), have allowed the detailed study of receptor types in the central nervous system and the definition of an increasing diversity of receptor sub-types (Creese, 1981; Haigler, 1981; Minneman, 1981). These developments also provide information on the mechanisms which translate receptor activation into intra-cellular effects.

The main steps in the pharmacodynamic phase of action of bioactive agents, including neurotransmitters, can be summarised as follows;



The initial step, involving the interaction of the ligand with the receptor site, is quantified by application of the law of mass action(Ariens , 1979) and can be expressed in the most general form as:

Equation 1:

$$L+R \underset{K_{-1}}{\overset{K_1}{\rightleftharpoons}} LR \underset{K_{-2}}{\overset{K_2}{\rightleftharpoons}} LR^* \underset{K_{-3}}{\overset{K_3}{\rightleftharpoons}} L+R^* \underset{K_{-4}}{\overset{K_4}{\rightleftharpoons}} \underline{R} \underset{K_{-5}}{\overset{K_5}{\rightleftharpoons}} R$$

Where L is the ligand, R is the receptor in its non-activated state, R* the receptor in the activated state, and $\underline{R}$ the receptor in a non-receptive state. The equilibrium constants governing the relative amounts of the different states are represented by K_x. The receptor complex LR* and the R* receptor contribute to the events leading to the effect. The $\underline{R}$ state is included to explain such phenomena as tachyphylaxis and specific desensitisation after repeated exposure to the ligand.

In the case of rapid receptor regeneration, equation 1 can be reduced to that for the occupation-activation model of receptor function (equation 2), which is closely related to the classical Michaelis-Menton equation for substrate-enzyme interaction (Ariens and Beld, 1977; Ariens et al. 1979).

Equation 2:

$$L+R \underset{K_{-1}}{\overset{K_1}{\rightleftharpoons}} LR \xrightarrow{K_2} LR^* \xrightarrow{K_3} L+R$$

The stimulus generated is postulated to be proportional to the fraction of the receptors in the activated state (LR*). The alternative 'rate' model of receptor activation holds that the stimulus generated is proportional to the rate of receptor occupation (K_1) (Paton, 1961).

The occupation-activation model seems to be adequate for the explanation of most cellular interactions with endogenous transmitters. However, in this model the receptor is regarded as a single non-interactive entity, which may not be the case. Competitive antagonists at the alpha-adrenergic , H_1-histaminic, cholinergic, dopaminergic and 5HT receptors are predominantly hydrophobic, whereas the appropriate agonists are polar in nature. Study of the structure/activity relationship between these compounds clearly indicates that the antagonists predominantly bind to sites accessory to those for the corresponding agonists (Ariens et al. 1964 ; Ariens and Simonis , 1976). These findings have led to increasingly complex theories of receptor activation (Changeaux et al. 1967 ; Pert and Snyder , 1974;Cuatrecasas et al. 1975 ; review: Hollenberg ,1978).

Receptor activation usually triggers a secondary cascade of responses (the amplifier system) possibly by an allosteric interaction with other membrane components (Cuatrecasas, 1974).Stimulation of some receptors, such as the nicotinic cholinergic receptor, directly alters ionic gradients by affecting the properties of membrane ion channels (Heidman and Changeux, 1978). However, in many cases receptor activation causes a change in the intracellular level of a 'second messenger' substance. Membrane bound adenylate cyclases which are stimulated to produce cAMP by the specific activation of dopamine receptors (see Creese, 1981), β-adrenoceptors (see Minneman, 1981) and 5HT-receptors (see Fillon, 1983), have been described. Although alpha-adrenoceptor activation

has been demonstrated to influence intracellular cAMP formation, the effect is not consistent between all alpha-adrenoceptors. Other possible second messengers at these sites include calcium ions and the membrane lipid, phosphatidylinositol (Minneman, 1981). So it appears that a variety of second messengers can be produced by receptor activation and that a given receptor type is not always associated with the same second messenger.

Generally ,the second messenger can act directly, or through a further cascade process, to affect the activity of appropriate metabolic enzymes or the expression of particular genes (both possible effector systems in diagram above), to bring about a change in the activity of the cell.

Regulation of the process outlined in the above diagram is possible at any of the individual steps. The quantal relationship between receptor activation and the intracellular response may be altered to allow physiologically adaptive processes to occur. Also, and of prime importance in the present context, are the ways in which the combination of ligand and receptor may be subject to regulation. Control exercised at this level would also allow adaptive changes to occur in the post-synaptic cell,and could result in the differential secretion of a given hormone.

1.5.3. Receptor Regulation

Receptor regulation has been the subject of many recent reviews (e.g. Hollenberg, 1979; Fambrough, 1980; Chuang et al. 1984). Most reviewers identify two factors which may predominate in influencing the dynamics of receptor turnover in vivo : the influence of neurotransmitter activity and the influence of steroid hormones.

The phenomena of hypo- and hyper-sensitivity of excitable cells are well documented (see Fleming, 1976). These phenomena occur in response to chronic changes in stimulation as for example in the rat where an increase in the activity of noradrenergic input to the cerebral cortex has marked effects on β-adrenoceptor density (Sporn et al. 1977).

studies on the rat also provide examples of the influence of steroids on neurotransmitter function. For example, in ovariectomised rats, oestrogen treatment increases the levels of putative hypothalamic β-adrenergic, muscarinic, cholinergic and 5HT receptors (McEwen and Parsons, 1982). Dopamine receptors in the striatum of ovariectomised rats also increase after oestrogen treatment (Hruska and Silbergeld, 1980) and similarly, dopamine receptors in the pituitary gland are under oestrogenic control (Heiman and Ben-Jonathan, 1982).

The mechanisms by which these changes occur may alter the availability of receptors in the cell membrane in a number of ways. Receptor formation may be induced via a genomic action (McEwan and Parsons, 1982) or alternatively, receptor turnover may be related to alterations in the fluidity of the cell membrane (Heron et al. 1980).

These possible mechanisms controlling receptor regulation are additional means, apart from the regulation of neurotransmitter turnover, by which the functional activity of a given neurone system may be changed, and may be in part responsible for the endocrine changes associated with broodiness.

1.6. ANATOMICAL CONSIDERATIONS IN THE CONTROL OF ANTERIOR PITUITARY GLAND FUNCTION IN BIRDS

Any consideration of the mechanisms controlling hormone release from the anterior pituitary gland must take into account the anatomical organisation of the hypothalamo-hypophysial system. The purpose of this section is to summarise briefly the salient anatomical features of the avian hypothalamo-hypophysial complex with particular influence on the secretion of prolactin and luteinizing hormone.

1.6.1. The Link between the Hypothalamus and Pituitary Gland

The hypophysial portal system was first described in mammals by Popa and Fielding (1930). They observed a system of capillaries in the median eminence which united to form vascular trunks in the pituitary

stalk and broke up again into sinusoid capillaries in the anterior pituitary gland. The functional significance of this system was first realised by Harris (1948), who suggested that chemical mediators produced in the brain were transported by the hypophysial portal blood supply to influence specific aspects of anterior pituitary gland function. The hypophysial portal vasculature is now generally accepted as being the final link between the brain and anterior pituitary gland (review : Everett, 1978).

The avian hypophysial portal system has been described in the chicken (Green, 1951; Hasegawa, 1956) and reviewed by Mikami (1980). In birds the infundibular artery supplies the almost independent capillary plexus, corresponding to the anterior and posterior divisions of the median eminence, which converge to form the anterior and posterior groups of portal vessels. The anterior portal vessels, originating from the anterior median eminence, supply the sinusoids of the cephalic lobe of the anterior pituitary gland, while the posterior portal vessels, from the posterior median eminence, supply the sinusoids of the caudal lobe of the anterior pituitary gland.

1.6.2. Prolactin and Luteinizing Hormone in the Anterior Pituitary Gland.

The development of immunohistochemical techniques has permitted the identification and characterisation of cell types in the anterior pituitary of birds on the basis of the hormones they contain. The application of this technique shows that prolactin containing cells occur in the cephalic lobe of the anterior pituitary gland (McKeonin, 1972; Marchand et al. 1975; Hansen and Hansen, 1977), while LH-immunoreactive cells occur in both the caudal and cephalic lobes (Marchand and Sharp, 1977). Recently it has been demonstrated that many LH-immunoreactive cells contain FSH-immunoreactivity (Mikami, 1983).

1.6.3. The Avian Median Eminence

The median eminence consists of three layers, an inner ependymal layer an intermediate fibre layer and an outer palisade layer. The primary capillaries of the hypophysial-portal system are predominantly located at the surface of the median eminence, rarely penetrating the inner layers (Benoit and Assenmacher, 1951). Within the median eminence specialised ependymal cells (tanycytes) form a functional link between the third ventricle and the hypophysial portal vessels (Knigge and Silverman, 1972; Kobayashi et al, 1972). The major innervation of the median eminence is supplied by axons originating in two distinct regions of the hypothalamus. Classically, the anterior median eminence receives fibres mainly from the magnocellular neurosecretory cells of the paraventricular, supraoptic and pre-optic regions via the hypothalamo-hypophysial tract, while the posterior median eminence is mainly innervated by fibres from the nucleus infundibularis via the tubero-hypophysial tract (Oksche and Farner, 1974). Although the LHRH cells are in the anterior hypothalamus (Sterling and Sharp ,1982), and the VIP cells are in the posterior basal hypothalamus they both innervate ~~anterior and posterior median eminence innervate both~~ both parts of the median eminence (section 7).

A rich monoaminergic innervation of the median eminence has been described (Calas et al. 1974) and numerous neuro-endocrine peptides have been localised in nerve terminals in the median eminence (see Blahser, 1983). The anatomical diversity of input to the median eminence and the neurochemical diversity of substances are consistent with an integrative function in the control of anterior pituitary function.

1.6.4. The Hypothalamus

The avian hypothalamus is heavily innervated by both monoaminergic and peptidergic neurones (Oksche, 1983) which may be directly or indirectly involved in the regulation of neuroendocrine function.

Primary control of LH secretion resides in the LHRH neuronal system which has been described in the chicken by Sterling and Sharp (1982) and Jozsa and Mess (1982). The cell bodies of this system are principally located in the anterior hypothalamus close to the midline. Fibres from these cells pass through the basal hypothalamus to terminate in the external layer of the median eminence. The neurone system primarily concerned with the stimulation of prolactin secretion has not yet been identified, although evidence cited in section 1.2. suggests that the avian prolactin - releasing factor is not a monoamine.

The only major grouping of monoamine containing cells intrinsic to the avian hypothalamus occurs in the paraventricular organ (Sharp and Follett, 1968). These cells, which are thought to possess sensory function, are exposed to the cerebrospinal fluid of the third ventricle, and contain dopamine and 5-hydroxytryptamine (Vigh and Vigh-Teichmann, 1973). Neuronal projections extending from the paraventricular organ into the surrounding basal hypothalamus may be the origin of some of the dopamine and 5-hydroxytryptamine containing terminals in the median eminence (Oksche, 1983).

Although there is as yet no direct evidence of a functional link between the systems of peptidergic and monoaminergic neurones which innervate the hypothalamus, their overlapping distributions and the effects of pharmacological manipulations on hormone secretion (section 1.2 and 1.3), suggest that the LHRH and putative PRF systems may be influenced by such interactions.

1.7. EXPERIMENTAL OBJECTIVES

Because of the large species and strain differences in factors which influence prolactin secretion (section 1.2), the primary objective of this study was to achieve a better understanding of the neuroendocrine mechanisms controlling broodiness in the bantam hen. To this end the following investigations were undertaken:

1. The anatomical distribution of 5-hydroxytryptamine-containing neurones in the bantam brain was assessed immunohistochemically, and the distribution of monoamine-containing neurones in the bantam hypothalamus was assessed by a fluorescence method.

2. The functional activity of monoaminergic neurones in the hypothalamus was compared in laying and broody bantams using a combination of techniques based on the measurement of monoamines by high performance liquid chromatography coupled with electrochemical detection.

3. The distribution and changes, associated with endocrine state, of putative 5-hydroxytryptamine and dopamine receptors in the pituitary gland and hypothalamus of out-of-lay, laying and broody bantam hens, were assessed by radioligand binding studies in vitro.

4. Various pharmacological treatments were employed in vivo and in vitro to assess the role of 5-hydroxytryptamine in the control of prolactin secretion in the bantam hen.

5. The anatomical distribution of neurones in the bantam hypothalamus containing vasoactive intestinal polypeptide(VIP)-like material, was determined using an immunohistochemical technique. Further, the possible physiological role of VIP on prolactin release was assessed both in vivo and in vitro .

2.0:MATERIALS AND METHODS

2.1 ANIMAL HUSBANDRY AND SURGERY

Bantam hens and cockerels were bred at the Poultry Research Centre from a strain selected to incubate game-bird eggs at the Institute of Terrestrial Ecology, Banchory. The birds were sexed at 24 weeks of age and the hens were transferred to individual battery cages (46 x 30 x 40 cm), exposed to 18 hours of light per day and allowed free access to commercial layer mash and water. The temperature in the hut was regulated to 21°C. Only hens laying five eggs on successive days were classified as 'laying' for experimental purposes.

Regression of the ovary and oviduct in laying hens was induced by decreasing the photoperiod to 8 hours of light per day. After 4-6 weeks of exposure to short days most birds had stopped laying. Those that had stopped and whose ovary and oviduct were found to be regressed on post mortem examination were used in studies requiring non-laying hens which were not broody.

Incubation was induced by transferring groups of 4-6 laying hens to deep-litter floor pens (2 x 1.5 m) containing 6 nest boxes. Hens were allowed to accumulate clutches of eggs (6-10 eggs per bird). The temperature in the pens was regulated to 21°C and the photoperiod was 18L:6D. Hens that had been sitting continuously on eggs for 7-14 days were classified as 'incubating'.

Nest-deprived-incubating (NDI) hens were used in some studies. Two procedures were used for nest deprivation; incubating hens were removed from their nest-boxes and allowed to remain in the pens, the nest-boxes being sealed with heavy card. Alternatively, incubating birds were removed from their nests and transferred to individual wire battery cages.

Surgical ovariectomies were carried out under general anaesthesia (Equithesin) at about 12 weeks of age while the hens were still sexually immature. The ovary was removed through an incision between the last two ribs, and the surrounding tissue destroyed by cautery. The site of the contra-lateral gonad was also cauterized to prevent the development

of an ovotestis. Ovariectomized birds were housed in cages and exposed to an 18L:6D photoperiod until they reached somatic maturity. Birds showing comb growth, which indicated ovarian development, were culled.

2.2 BRAIN DISSECTIONS

Birds were usually killed by cervical dislocation and decapitated. The lower mandible and associated musculature was removed followed by the upper mandible forward of the frontal process. This allowed the optic nerves to be cut and the eyes to be removed. The entire ventral aspect of the cranium was exposed by removing the musculature attached to the basisphenoid, the presphenoid of the sphenoidal bone and the pterygoidal bone. The ventral surface of the brain was exposed by careful removal of the covering bone. Particular care was taken when removing the bone surrounding the sella turcica to ensure the pituitary gland was not damaged.

For histological studies the whole brain was removed from the skull. Further dissection took place in a glass petri dish on ice and consisted of the excision of parts of the mid and hind-brain to give manageable 'blocks' for processing (usually 5-8 mm cubes).

For other studies the pitutary gland was carefully removed and neurohypophysis cut away from the median eminence. The optic chiasma was then removed, care being taken not to damage the median eminence. The hypothalamus, including the median eminence, was removed as one piece by two cuts parallel with the midline (2.0 - 2.5 mm lateral) a transverse cut at the level of the pre-optic recess, a transverse cut at the posterior margin of the median eminence, and a final cut parallel with the ventral surface of the hypothalamus at a depth of 4.0 - 5.0 mm. When necessary, the hypothalamus was further divided into anterior and posterior portions by a transverse cut midway through the supraoptic decussation.

The full dissection was completed within 5 minutes of death. Pituitary glands, anterior hypothalami and posterior hypothalami destined for neurotransmitter binding site studies and monoamine determination, were

immediately frozen in liquid nitrogen in individual sealed tubes (Sarstedt Ltd) and stored at -80°C for no more than 20 days before use. The pituitary glands and posterior hypothalami for use in in vitro incubations were stored on ice in incubation medium for no more than 30 minutes prior to use.

2.3 HISTOLOGY

An immunohistochemical technique (modified from Hsu et al. 1981) was used to locate and map the distributions of 5-hydroxytryptamine and a vasoactive intestinal polypeptide - like substance in brain tissue. The distribution of the biogenic monoamines was also examined using the sucrose-phosphate-glyoxylic acid induced fluorescence method of De La Torre and Surgeon (1976)

2.3.1 Tissue Preparation for Immunohistochemistry

The birds were deeply anaesthetized with Sagatal (May and Baker Ltd) and the carotid arteries were cannulated in the direction of the head. Approximately 4 mls of heparinized 0.75% saline (1000 I.U. per ml, Boots the Chemist) was infused through the cannuli shortly before both jugular veins were severed. The head was then perfused under pressure with approximately 200 mls of 0.75% saline to clear the perfusion 'loop' of blood. This was followed by 200-300 mls of Bouin's solution without acetic acid. The perfusion was considered to be complete when the wattles and comb assumed the deep yellow colour of the fixative. The brain was then removed as described earlier (section 2.2) and selected areas excised and fixed for a further 1-4 hours in Bouin's solution.

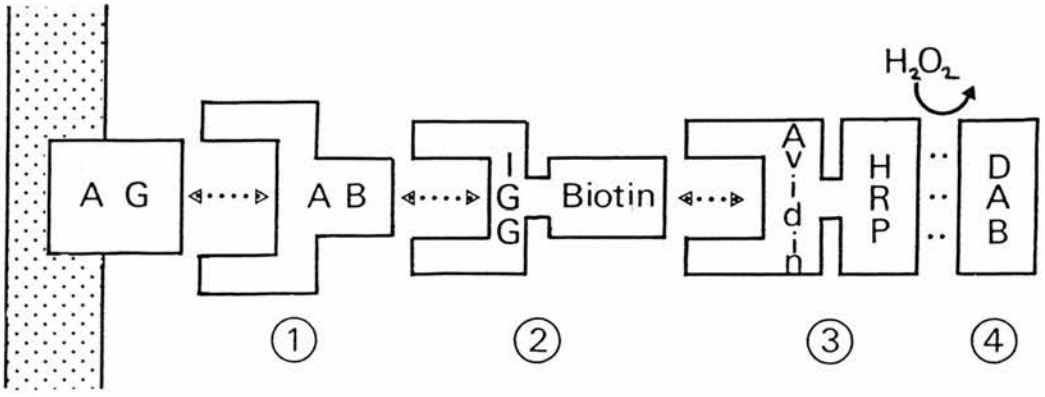
Some blocks of brain tissue were cut on a Vibratome (Oxford Instruments Ltd) without further processing and the sections (15-25 μm) were immediately subjected to the immunohistochemical procedure. Most tissues were dehydrated through a graded series of alcohols (4 x 30 minutes in 70, 90, 95 and 100% ethanol), and then exposed to several changes of xylene until cleared.

Wax embedding was preceded by four 30 minute incubations in liquefied paraffin wax (60°C, Histo-Lab Ltd). The resulting blocks were trimmed and mounted on microtome chucks and stored at 4°C.

Serial sections of 5-10μm , thickness were cut from the wax embedded tissue on a bench-top microtome (model AO 820, American Optical Corp.). Selected sections were floated in a water bath at 58°C and mounted on cleaned glass microscope slides coated with a chrome alum/gelatin mixture. Mounted preparations were dried for 18-24 hours at 30°C and stored at 4°C until processed.

2.3.2 Immunohistochemical Procedure

The immunological steps used in the localisation of a tissue antigen can be schematically represented as follows;



The primary antibody (1) - antigen complex is bound by a second antibody (2).This second antibody is an immunoglobin which has had the vitamin biotin, chemically incorporated into it. The bound biotin moieties are then recognised and bound by the glycoprotein avidin, which is added in a complex with horseradish peroxidase (3). The tertiary complex thus formed is made visible, for light microscopy, by incubation in a peroxide substrate solution (in this case, diaminobenzidine) in the presence of H_2O_2 which produces a stable coloured product.

The procedure used was modified from that of Sternberger et al. (1970) and was applied to both mounted and free floating sections. Two buffers were used in the following procedure; PBS: 50 mM phosphate buffered 0.9% saline, titrated with 0.2 N NaoH to pH 7.4 at 4°C, and Tris-HCl.: 50 mM Tris titrated with 1N HCl to pH 7.6 at room temperature.

1. Dewax in xylene followed by absolute ethanol.
2. 30 minutes in 0.5% H_2O_2 in absolute methanol to remove endogenous peroxidase.
3. Hydration through a graded series of alcohols followed by washing in PBS at 4°C.
4. 10 minutes in 0.4% Triton-X-100 (Koch-Light Laboratories Ltd) in PBS at 4°C to facilitate antibody penetration.
5. Wash in PBS at 4°C.
6. 30 minutes in 20% normal goat serum (Scottish Antibody Production Unit, SAPU),in PBS at 4°C, to reduce non-specific background staining.
7. 18-24 hours in appropriately diluted primary antibody (raised in rabbit) in PBS at 4°C.
8. Wash in PBS at 4°C.
9. 30 minutes in biotinylated rabbit immunoglobin-G (Vector Laboratories Ltd), 1:400 in PBS at 4°C.
10. Wash in PBS at 4°C.
11. 60 minutes in avidin-horse radish peroxidase complex (Vector Laboratories Ltd), 1:250 in PBS at 4°C.
12. Wash in PBS at 4°C.
13. Wash in Tris-HCl at room temperature.
14. 2-5 minutes in 0.05% diaminobenzidene plus 0.003% H_2O_2 in Tris-HCl at room temperature.
15. Rinse in tap water and either immediately mounted with glycerol/PBS solution, or dehydrated and mounted with DPX (BDH Ltd).

Steps 1-3 applied only to wax embedded preparations. Sections were processed in groups of 24 or 48. Each group contained control sections that were incubated with normal rabbit serum (SAPU) in the place of

primary antibody or were incubated with primary antibody which had been exposed to the putative antigen in solution for 24 hours at 4°C. No specific reaction product was found in control preparations.

2.3.3 Primary Antisera

The antiserum raised against 5-hydroxytryptamine (code no. 40122) was purchased from Immunonuclear Corporation, USA. It was produced in a rabbit against a 5-hydroxytryptamine-bovine serum albumin conjugate, and had been shown to localise specifically 5HT in known 5HT pathways and cell bodies in monkey, cat and rat brain. In the present study preincubation of the diluted antiserum with 200 mg ml^{-1} 5-hydroxytryptamine.HCl resulted in complete inhibition of staining. The antiserum was used at dilutions of between 1:200 and 1:1000 in PBS.

The antiserum raised against porcine vasoactive intestinal polypeptide (pVIP) was purchased from R.I.A.(UK) Ltd. It was raised in rabbit against pVIP conjugated to keyhole limpet haemocyanin-glutaraldehyde. Preincubation of the diluted antiserum with 2 µg ml^{-1} pVIP (Cambridge Research Biochemicals) resulted in complete inhibition of staining. The anti-VIP serum was used at dilutions of between 1:500 and 1:2000 in PBS.

2.3.4. Localisation of Staining

Precise anatomical identification of the sites of immunohistochemical staining was assisted by counter-staining with gallocyanin or crysol fast violet (Bancroft and Stevens, 1982). Observed specific staining was catalogued and plotted on transverse and para-sagittal representations of the chicken brain (van Tienhoven and Juhasz, 1962; Sterling and Sharp, 1982).

2.3.5 Monoamine Histochemistry

Appropriately dissected blocks of fresh brain tissue were embedded in cryostat embedding medium (Tissue-Tek II, Tissue-Tek Ltd). After equilibration in the cryostat cabinet (-20°C), serial sections of 20-30 µm thickness were cut and mounted on clean glass microscope slides.

Monoaminergic fluorescence was induced using the sucrose-phosphate-glyoxylic acid method of De La Torre and Surgeon (1976). Preparations were examined and photographed on a Zeiss-photomicroscope II with a fluorescent attachment. Specific reaction product was mapped on para-sagittal representations of the chicken brain after counterstaining with crysol fast violet.

2.4 ESTIMATION OF MONOAMINE CONCENTRATION AND TURNOVER IN BRAIN TISSUE

Monoamine neurotransmitter turnover is a dynamic equilibrium between synthesis, storage, release and metabolism. Turnover can therefore be assessed experimentally as a ratio between the parent monoamine and its major metabolite,using single point estimates. Alternatively the catabolism of the monoamine can be reduced by inhibitors of monoamine oxidase, and the resulting accumulation of monoamine in the tissue over time reflects the rate at which it is being produced, and by implication reflects the functional state of that aminergic neurone (see section 1.4)

Concentrations of the biogenic monoamines in samples of brain tissue were measured using high performance liquid chromatography (HPLC) with electrochemical detection. The method involves the separation of the constituents of the extracted sample by reverse-phase ion-pairing liquid chromatography prior to their oxidation at a glassy-carbon electrode. Differential column retention of noradrenaline (NE), adrenaline (A), dopamine (DA), 5-hydroxytryptamine (5-HT) and their major metabolites allowed their measurement in a single sample without elaborate extraction procedures.

Two groups of birds were used in each experiment. One was injected with 1 ml kg^{-1} 0.75% saline (i.p.) and the other with 75 mg kg^{-1} pargyline (Sigma) in 0.75% saline (i.p.). Pargyline is a reasonably specific monoamine oxidase inhibitor and blocks the major pathway of intraneuronal catabolism. In a pilot experiment (see section, 4.3.3) the accumulation of NE, A, DA and 5HT was found to be linear over the 2.25 hour following an injection of pargyline, and appeared to be optimal at 2 hours. Both groups of birds were killed 2 hours after

injection on the basis of the results of the pilot study. The hypothalami were rapidly excised (see section 2.2) and stored at -80°C until processed.

2.4.1 Tissue Preparation

Weighed anterior and posterior hypothalami were prepared for assay by sonication (10 seconds, 10 microns peak to peak) on ice in 150 µl of 0.3 M perchloric acid. Following centrifugation (10,000 xg for 5 minutes) the supernatants were collected and 20 µl aliquots analysed.

2.4.2 HPLC Instrumentation

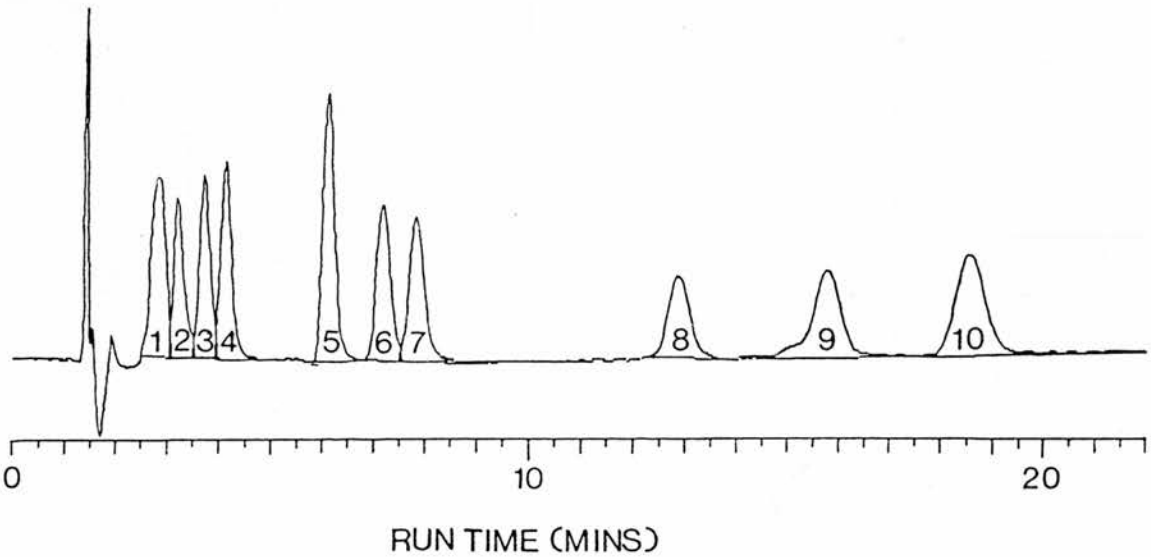
The chromatograph used in all determinations comprised a Gilson 302 pumping unit and a 20 µl injection head above an Altex ultrasphere-ODS ion-pairing chromatographic column (250 x 4.6 mm, 5 mm) coupled to an LC-17 glassy-carbon transducer governed by an LC-45 electronic controller (Bioanalytical Systems Inc.). The detector was run at 800 mV and had a sensitivity of 2nA full scale deflection. The flow rate was 1.6 ml min^{-1} generating a pressure of 3000 p.s.i.Each run lasted between 18 and 22 minutes.

2.4.3 Mobile Phases

The mobile phases were essentially the same as those described by Mayer and Shoup (1983):- Acetonitrile (Rathburn Chemicals Ltd) at a final concentration of 3.5% was added to ultrapure water containing 150 mM monochloroacetic acid, 120 mM sodium hydroxide, 1 µM 1-octanesulphonic acid and 0.6 µM Na_2EDTA (all chemicals were 'HPLC' grade supplied by Fisons Ltd). After filtering through a 0.2 µm pore membrane (Millipore Inc) and degassing, the mobile phases were completed by the addition of sufficient tetrahydrofuran (Rathburn Chemicals Ltd) to make a 1.825% (v/v) solution. After a further brief degassing the solution was capped at all times to prevent evaporation of the organic phases.

2.4.4 Standards

Noradrenaline hydrochloride, adrenaline hydrochloride, dopamine, homovanilic acid, 5-hydroxytryptamine, 5-hydroxyindole-3-acetic acid, 3,4-dihydroxyphenyl acetic acid, 5-hydroxyhyptophan and 4-methoxy-3-hydroxyphenyl glycol as the hemipiperazine salt, were obtained from the Sigma Chemical Company and were used as received. A standard solution containing 1000 pmols per ml of each solute (as a mixture) was made up in 0.3 M perchloric acid and 20 µl of this solution was used to calibrate the HPLC system. The result of a typical calibration run is given below.



2.4.5 Data Handling

Chromatographs resulting from injection of samples of unknown composition were analysed by calculation of peak area and retention time relative to a calibration run of known concentration by a Gilson 'Datamaster' data reduction unit, and were corrected for original wet weight of tissue.

Steady state estimations of all monoamines were calculated as the mean and standard error of the mean, in groups of 8 birds killed two hours after injection with saline. The difference between the steady state estimate and that from groups of 8 birds killed two hours after injection with pargyline, was the turnover index.

2.5 RADIOLIGAND BINDING STUDIES

The binding of ^{3}H-lysergic acid diethylamide to putative 5-hydroxytryptamine receptors and ^{3}H-domperidone to putative dopamine receptors in preparations of hypothalami and pituitary glands was assessed in vitro.

2.5.1 Tissue Preparations

Brain tissue from non-laying , laying, incubating and p-chloramphetamine treated (see section 2.6.3) bantam hens was collected and stored as described in section 2.2, and processed in one of the following ways;-

1. It was homogenized in 10-20 volumes of ice-cold 0.32 M sucrose, followed by centrifugation (4°C) at 1000 x g for 10 minutes to yield the crude nuclear pellet (P1). The crude mitochondrial pellet (P2) was then isolated from the supernatant by centrifugation (4°C) at 20,000 x g for 10 minutes. The P2 was lysed by homogenization in 10-15 volumes of ice-cold H_2O and centrifuged at 8000 x g for 30 minutes. The supernatant and soft upper 'buffy coat' of the residual P2 pellet (RP2) were collected and centrifuged (4°C) at 100,000 x g for 20 minutes to yield the crude synaptic membrane pellet (CSM). P1, RP2 and CSM pellets were superficially washed with water and resuspended in the appropriate incubation buffer by gentle homogenisation.

2. It was disrupted in 20-50 volumes of ice-cold buffer A (see below). using a polytron (setting 5 for 20-30 seconds). Total particulate material was isolated by centrifugation (4°C) at 50,000 x g for 30 minutes. The pellet was washed twice in buffer A by rehomogenisation and centrifugation. Between washes the homogenate was incubated for 10-15 minutes at 30°C. After the second wash the pellet was resuspended in the appropriate incubation buffer (B or C).

The buffers used were the following;

Buffer A : 50mM Tris-HCl pH 7.4 at 4°C

Buffer B (for ^{3}H-LSD studies): 50mM Tris-HCl containing 2.7 mM $CaCl_2$, 9μM pargyline and 0.1% (w/v) ascorbic acid. The final pH was adjusted to 7.4 at 40°C with 1N HCl.

Buffer C (for ^{3}H-domperidone studies): 50 mM Tris-HCl containing 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 μM pargyline and 0.1% (w/v) ascorbic acid. The final pH was adjusted to 7.4 at 37°C with 1N HCl.

All buffers were prepared immediately prior to use in clean glassware using ultrapure water (Millipore MX water purifying unit)

2.5.2 Binding Experiments

Preliminary experiments were performed to ascertain the degree of specific binding to various tissue fractions, to establish the relationship between binding capacity and tissue/protein concentration, and to optimise incubation time and temperature. These experiments were performed for both radio-ligands using homogenised whole hypothalami from laying hens.

Using the preliminary data to define the assay conditions two types of binding experiment were routinely performed:

1. Saturation studies in which known concentrations of radioligand in the nano-molar range were incubated in triplicate with a fixed concentration of prepared tissue. The non-specific binding component at each concentration of radioligand was also determined in triplicate in a parallel incubation in the presence of an excess of the unlabelled ligand.

2. Displacement studies in which the specific binding of a single known concentration of radioligand (close to the estimated K_D) to a fixed amount of tissue, and the ability of an unlabelled test ligand to inhibit this binding, was determined.

2.5.3 ^{3}H-LSD Binding Protocol

The experimental protocol for ^{3}H-LSD binding studies (modified from Pert and Snyder, 1973) required the estimation of total binding (TB), non-specific binding (NSB), and displacement of total binding by test ligands (DTB). All estimates were made in triplicate and carried out in clean glass tubes (9 x 60 mm) containing the following;

Total binding: 100µl of ^{3}H-LSD, 100µl of Buffer (B) + 500 µl of tissue homogenate.

Non-specific binding: 100 µl of ^{3}H-LSD + 100 µl of 0.7 µM LSD + 500 µl of tissue homogenate.

Displaced total binding: 100 µl of 24 nM ^{3}H-LSD + 100 µl of test ligand + 500 µl of tissue homogenate.

For saturation studies, both TB and NSB were estimated for each concentration of ^{3}H-LSD used. For displacement studies ^{3}H-LSD at a final concentration of 4nM was used while the concentration of test ligand was altered.

(N-methyl-^{3}H) lysergic acid diethylamide was purchased from New England Nuclear (W Germany) and had a specific activity of between 50 and 60 Ci $mmol^{-1}$ depending on batch. Lysergic acid diethylamide as the tartrate salt (LSD) was purchased from Sandoz Pharmaceuticals Ltd (London). Test ligands used were LSD, quipazine (Miles-Davis Ltd), cyproheptadine (Janssen Phamaceuticals Ltd), 5-hydroxytryptamine and dopamine (both Sigma). Test ligands were dissolved in buffer B. Quipazine and cyproheptadine were initally dissolved in acidified H_2O.

Ice-cold tissue homogenate was added to previously prepared tubes containing the appropriate reagents at 4°C. The contents were mixed on a rotamixer and placed in a shaking water bath at 40°C. The incubations were terminated after 30 minutes by the addition of 5 ml of ice-cold buffer A and rapid filtration. Glass fibre filters of 16 µm pore size (GFA, Watman Ltd), soaked for 10-24 hours in 0.1 µM LSD, were used in a vacuum filtration manifold (VFM3, Amicon Ltd). Following filtration, the samples were washed with 4 x 10 mls of ice-cold buffer A (10-15

seconds). The filters were air dried for 1-2 hours at room temperature and then placed in scintillation vials containing 1 ml of protein digestant (Protosol, New England Nuclear Ltd). The capped vials were left at room temperature overnight before neutralisation with 50 µl of glacial acetic acid. After the addition of 10 ml of scintillation cocktail (Fisoflour 1 MPC, Fisons Ltd), and several hours of equilibration in the dark, the samples were counted on a Beckmann LS-230 β-counter.

2.5.4 ^{3}H-Domperidone binding Protocol

A similar procedure was used to assess ^{3}H-domperidone binding (modified from Baudry et al. 1979). All estimates were made in triplicate and carried out in clean glass tubes (9 x 60 mm) containing:

Total Binding (TB): 100µl ^{3}H-domperidone + 100 µl buffer C + 200 µl tissue homogenate.

Non-Specific Binding (NSB): 100 µl of ^{3}H-domperidone + 100 µl of 4 µM domperidone + 200 µl tissue homogenate.

Displaced Total Binding (DTB): 100ul of 16 nM ^{3}H-domperidone + 100 µl test ligand + 200 µl tissue homogenate.

For saturation studies TB and NSB were estimated for each concentration of ^{3}H-domperidone. A final concentration of 4 nM ^{3}H-domperidone was used in all tubes for displacement studies while the concentration of test ligand was altered.

(Benzene ring-^{3}H)-domperidone was purchased from New England Nuclear(W. Germany) and had a specific activity of between 30 and 35 Ci mmol^{-1}. Domperidone was the kind gift of Janssen pharmaceuticals Ltd. Test ligands used were domperidone, (+) butaclamol (Research Biochemicals Inc. USA) (-) butaclamol (Ayrst pharmaceuticals Ltd) apomorphine, 5-hydroxytryptamine, dopamine (all from Sigma Ltd.) and methysergide (Sandoz Products Ltd). Domperidone, butaclamol and apomorphine were dissolved in 0.7% lactic acid prior to dilution in buffer C.

The incubations lasted 30 minutes and were maintained at 37°C in a shaking water bath. They were terminated by the addition of 5 ml of ice-cold buffer A and rapid filtration followed by 3 x 5 ml washes with ice-cold buffer A. Samples were prepared as before for β-counting. The filters were pre-soaked in 1 μM domperidone for 16-24 hours prior to use.

2.5.5 Blanks

Experimental blanks were included in all binding studies. These took the form of tubes in which the homogenate was replaced with buffer. The binding of radioligands to the filters was less than 1% of total bound activity. Counting efficiency was periodically calculated by the addition of a known amount of ^{3}H-Hexadecane to a previously counted sample.

2.5.6 Protein Determinations

Protein concentrations in tissue homogenates were determined by a modification of the Coomassie Brilliant Blue method (Bradford, 1976). Rabbit gamma-globulin (Sigma) was used as the protein standard over a range of 1-25 μg ml^{-1}. Concentrated Coomassie Brilliant Blue G-250 dye reagent (0.2 mls, Bio-Rad Labs Ltd) was mixed with 0.8 mls of standards and appropriately diluted samples in clean glass test tubes. Reagent 'blanks' contained 0.8 mls of the appropriate buffer. After 15-30 minutes the optical density at 395 nm was measured on an SP500 spectrophotometer (Pye-Unicam). Unknown sample potencies were calculated using the equation derived from linear regression of the optical density versus concentration of standards.

2.5.7 Calculation of Specific Binding

After the subtraction of 'blank' values from the raw data, the values for specifically bound counts were calculated by the subtraction of the means of triplicate estimates of total binding and non-specific binding. The value for specifically bound counts was converted to concentration of ligand specifically bound per unit protein in the following manner;

$$B = \frac{CPMSP}{60} \times \frac{100}{E} \times \frac{1}{SA} \times \frac{1}{P}$$

Where B is bound ligand (fmol) per milligram protein, CPMSP is the estimate of specific counts bound, E is the percentage efficiency of the counter, SA is the specific activity of the radioligand (Bq f mol^{-1}) and P is the amount of protein present in the incubation in milligrams.

2.5.8 Scatchard and Hill Analysis

Throughout these studies the maximum tissue binding of radioligand represented less than 8% of the total amount of radioligand added to the incubation. Loss of up to 10% of the radioligand from the incubation system through tissue binding has been shown to have no significant effect on estimates of binding constants, when the added concentration of radioligand is taken as equal to the actual free ligand at equilibrium (Wells et al. 1980; De Lean et al. 1981). For the present purposes the concentration of free radioligand at equilibrium was taken as equal to the concentration of radioligand added.

Linear regression by the method of least squares was performed to give the line of best fit for a plot of bound/free versus bound radioligand. The slope of this line is equal to the negative reciprocal of the equilibrium binding constant (K_D), and its abscissa intercept is equal to the apparent maximum number of binding sites (Bmax).

This follows from the Scatchard equation (Scatchard, 1949) which can be represented as:

$$\frac{B}{F} = \frac{B\ max - B}{K_D}$$

From a plot of $\log_{10}$ (B/Bmax -B) versus $\log_{10}$Free the Hill binding constant (K_D^1) an alternative estimate of K_D, is the abscissa value where Log 10 (B/Bmax -B) equals 0. The slope of the line of best fit

calculated by linear regression gave the Hill number (Hn) which is a measure of binding homogeneity. If The Hn is unity, provided there is no cooperativity, there is a single homogeneous class of binding sites and the law of mass action is obeyed (Koshland, 1975). The Hn is calculated from the following equation;

$$\log \frac{B}{(Bmax-B)} = n \log (L) - \log K_D'$$

where n is the theoretical number of ligand binding sites per receptor molecule, K_D' is a composite constant of the K_D and interaction factors that determine the degree to which K_D is altered by each discrete binding step, and (L) is the concentration of radioligand.

In analysis of data from displacement studies, the difference between total CPM bound in the presence of test ligand and non-specific CPM bound, expressed as a percentage of specific CPM bound, allowed the construction of displacement curves. The data was also plotted as log %B max/(100-%Bmax) versus log (D) in a modification of the Hill plot, where (D) is the concentration of test ligand.

All data analysis was performed by computer using the Minitab data processing package.

2.6 PHARMACOLOGICAL TREATMENTS IN VIVO

Various drugs were tested for their ability to influence prolactin and luteinizing hormone secretion in laying and broody bantam hens. Most of the studies were short-term and all involved blood sampling by venepuncture prior to and at intervals following drug administration. Plasma samples were stored at -20°C until assayed for prolactin content in homologous and /or heterologous radioimmunoassays and for luteinizing hormone in a homologous radioimmunoassay.

2.6.1 Manipulation of 5-Hydroxytryptamine Function

The following drugs and some combinations thereof were all injected intraperitoneally, while a control group received an injection of vehicle;

DRUG	NOMINAL ACTIVITY	SOURCE	VEHICLE
5-hydroxytryptophan	5HT precursor	Sigma	Acid Saline
Quipazine	5HT receptor agonist	Miles-Davis	Acid Saline
Cyproheptadine	5HT receptor blocker	Jannsen Ph.	DMSO/Saline
SQ 10631	5HT receptor blocker	Squibb Ph.	DMSO/Saline
Methysergide	5HT receptor blocker	Sandoz Ph.	Saline
Fluoxetine	5HT-reuptake inhibitor	Sigma	Saline

2.6.2 Vasoactive Intestinal Polypeptide.

The influence of porcine vasoactive intestinal polypeptide (pVIP Cambridge Research Biochemicals) on prolactin, luteinizing hormone and growth hormone secretion was examined. Blood samples were taken prior to and at intervals after intra-venous injection of pVIP or saline. Plasma samples were stored at -20°C until assayed for prolactin, luteinizing hormone and growth hormone by radioimmunoassay. Growth hormone determinations were performed by Dr. S. Harvey of the Wolfson Institute, University of Hull.

2.6.3 p-Chloramphetamine Treatment

The selective 5-hydroxytryptamine neurotoxin ,p-chloramphetamine (Sigma) was administered to laying birds (20 mg kg^{-1} in 0.75% saline, i.p.) every second day for 13 days.Another group of birds was injected with an equivalent volume of saline on the same schedule. Groups of treated and control birds were killed on day 14 and day 21. The hypothalamic content of monoamines in these birds was measured by HPLC with electrochemical detection to assess the efficacy of the treatment. Most of the birds were killed by cervical dislocation on day 22 and their hypothalami were taken for a series of ^{3}H-LSD binding studies.

2.7 PHARMACOLOGICAL MANIPULATIONS IN VITRO

The effect of vasoactive intestinal polypeptide and the 5HT receptor agonist quipazine on prolactin release from the pituitary gland, was assessed using an in vitro incubation system modified from Border and Chadwick (1977) and Harvey et al. (1979). Aliquots of the resulting incubate were measured for prolactin content by radioimmunoassay and also analysed by polyacrylamide gel electrophoresis.

2.7.1 Tissue Collection

Anterior pituitary glands and posterior hypothalami were rapidly removed from birds killed by cervical dislocation. They were stored on ice in incubation medium for up to 30 minutes. The glands were then bisected longitudinally and the blotted halves weighed.

2.7.2 Incubations

Hemipituitary glands and posterior hypothalami alone and together were incubated in 200 ul of normal medium or medium containing the test drug. The incubations were performed in medium 199 with Earle's salts and sodium bicarbonate (0.22%) (Flow laboratories, Ltd) containing lmg ml^{-1} glutamine, 0.1 mg ml^{-1} penicillin, 0.05 mg ml^{-1} streptomycin, at 39°C over 4 hours and were continuously gassed with a 95% CO_2/5% O_2 mixture. After incubation the tubes were placed on ice, the medium rapidly aspirated and stored at -20°C until assayed. Porcine vasoactive intestinal polypetide (Cambridge Research Biochemicals) and quipazine were dissolved in incubation medium on the day of the experiments and were used at a concentration of 10^{-7} mole/l.

2.8 POLYACRYLAMIDE GEL ELECTROPHORESIS

Various protein constituents of post-incubation media and aqueous extracts of anterior pituitary glands and neurohypophyses were separated by non-dissociating discontinuous polyacrylamide gel electrophoresis (modified from Hames, 1981).

2.8.1 Reagents

The following stock solutions were prepared in clean glassware using ultrapure water and 'electrophoresis' grade chemicals (Fisons Ltd).

A. Resolving gel buffer: pH 8.9, 36.3 g Tris + 48 mls 1M HCl in 100 ml H_2O.
B. Stacking gel buffer: pH 6.9, 6.0 g Tris titrated with 1M HCl in 100 ml H_2O
C. Acrylamide-bisacrylamide: 28 g acrylamide + 0.7 g N,N',-methylene bisacrylamide in 100 ml H_2O
D. Acrylamide-bisacrylamide: 10 g acrylamide + 2.5 g N,N',-methylene bisacrylamide in 100 ml H_2O
E. Sucrose solution: 40 g sucrose in 100 ml H_2O
F. Persulphate solution: 0.28 g ammonium persulphate in 100 ml H_2O
G. Running buffer: (pH 8.3) 14.1g glycine + 3.0 g Tris in 5000 ml H_2O
H. Staining solution: 0.1% Coomassie Brilliant blue R250 in a 5:5:2 mixture of H_2O: methanol: glacial acetic acid
I. Destaining solution: 30% methanol + 10% acetic acid in H_2O

2.8.2 Gel Preparation

Slab gels, equivalent in nature to those used in a prolactin assay based on tube gel electrophoresis (see Nicoll and Nichols, 1971), were prepared. Low porosity (7.5%) resolving gel was made by mixing part for part; 1 x A, 2 x C, 1 x H_2O, 4 x F and 2.3 µl of tetramethylethylenediamine (TEMED, Fisons Ltd) per ml of A. High porosity (3.5%) stacking gel was made by mixing, 1 x B, 2 x D, 3 x E, 2 x F and 5 µl of TEMED per ml of B. Both mixtures were kept on ice until the gels were cast.

Using a 16 x 14 cm slab gel casting stand (Bio-Rad Labs Ltd), the resolving gel (14 x 12 x 0.3 cm) was overlayed by a stacking gel (14 x 4 x 0.3 cm). Sample wells of 200 µl volume were created in the stacking

gel by a 20 tooth teflon comb. Each sample (25 µl) was mixed with 175 µl of stacking gel containing 0.002% Bromophenol Blue and layered into individual sample wells.

2.8.3 Electrophoretic Conditions

The completed gels were placed in a water cooled slab gel cell (Protean, Bio-Rad Labs Ltd). Samples were stacked at a constant current of 20 mA supplied by an LKB 2103 power supply. Once the proteins had migrated into the resolving gel the system was run at a constant current of 50 mA until the ion front, marked by the tracking dye, migrated 10 cm towards the anode.

2.8.4 Post-electrophoretic Processing

When electrophoresis was completed, the gels were rapidly removed from the tank and either immersed in staining solution (H) for 16 hours at room temperature, or selected 'tracks' were cut from the body of the gel and divided into 0.5 cm sections from the ion front to the origin. Protein bands were differentiated in the stained gels by repeated immersion in destaining fluid (I). The sections of cut gel were homogenised in 1 ml of 0.5 mM phosphate buffered saline (pH 7.4) and left at 4°C for 48 hours to allow the protein to elute. After centrifugation (4°C, 1000 xg) the supernatant from each gel segment was assayed for prolactin content in the homologous radioimmunoassay. Stained gels were photographed and the negatives analysed by densitometry using a scanning densitometer (DD2,Kipp and Zonen Ltd). Micrograph records of densitometric scans were analysed using a MOP/AMO2 digitiser (Kontron, FGR) linked to a Primos computer package (created by Dr J Filshie of the PRC) for band RF values and peak area.

2.9 HORMONE DETERMINATIONS

Prolactin and luteinizing hormone radioimmunoassays, performed on plasma and samples from <u>in vitro</u> studies, were of the double antibody post-precipitation type and shared a similar protocol. Growth hormone

determinations were performed by Dr S Harvey of the Wolfson Institute (University of Hull) using a homologous chicken growth hormone radioimmunoassay (Harvey and Scanes, 1975).

2.9.1 General Assay Protocol

1. Total counts (x3); tubes containing ^{125}I-hormone only
2. Blanks (x3); tubes containing all reagents except first antibody and hormone
3. Zero standard(x6);tubes containing all reagents except unlabelled hormone
4. Standards (x3); tubes containing all reagent and known concentrations of hormone
5. Control sample (x3); tubes containing all reagents and the relevant plasma pool at various dilutions
6. Unknown samples (x2); tubes containing all reagents plus appropriately diluted experimental samples

2.9.2 Assay Procedure

Day 1. Standards and unknown samples were diluted and dispensed into assay tubes.Diluent was added at the appropriate volume to'Blank' and 'Zero Standard' tubes.First antibody was added to all tubes except 'total counts' and 'blanks'.The contents were mixed using a rotamixer and incubated at 4°C for 24 hours.

Day 2. ^{125}I-hormone was added to all tubes, contents were mixed and incubated for 24 hours at 4°C.

Day 3. Second antibody (precipitating serum) was added to all tubes.The contents were mixed and incubated at 4°C for a further 24-48 hours

Day 4. All tubes except 'total counts' were centrifuged (4°C) at 1500 xg for 40 minutes. Starch solution (50 µl of 5% w/v) was added to all tubes and they were spun for a further 10 minutes. The supernatant was then removed by a suction pipette.

All tubes were counted for 100 seconds on a Wallac 80,000 Gamma Sample Counter (LKB Ltd).

All samples and reagents were dispensed using a Microlab M diluting dispenser (Hamilton Ltd) into disposable polystyrene tubes (30 x 6 mm). Assay diluent was 0.04 M Na_2PO_4 containing 0.15 M NaCl, 8 mM EDTA, 2% normal horse serum and 0.1% (w/v) NaN_3. 1M NaOH was used to titrate the diluent to the required pH.

2.9.3. Assay Parameters

RIA:	**Homologous Prolactin**	**Heterologous Prolactin**	**Homologous LH**
Key Ref:	Scanes et al. ('76)	McNeilly et al.('78)	Follett et al.('72)
Diluent pH:	7.5	7.5	7.0
Hormone:	Chicken Prolactin Scanes et al.'75	Ovine Prolactin LER-800-2 (NIH)	Chicken LH (AE1) Scanes et al.'72
Iodination:	Iodogen	Lactoperoxidase	Chloramine T
Separation:	G15/25 & G100	G100	CF 11
Standards: (ng/ml)	1.65 - 200	0.8 - 100	0.12 - 7.0
Standard/Sample Volume:	100 µl	40 µl	200 µl
Sample dilution:	1:1/1:10/1:100	1:1/1:10/1:100	1:25/1:10/1:100

First Antibody:

anti-$_{ch}$PRL 5/5 50 µl	AGP-4, 20 µl at	CM2 15/8, 50µl at
at 1:3000 (1:300 NRS)	1:9000	1:20 000

^{125}I-Hormone:

Chicken PRL	Ovine PRL	Chicken LH
50 µl containing	20 µl containing	50 µl containing
5000 CPM	10,000 CPM	8000 CPM

Second Antibody:

50 µl DRPS (1:20)	20 µl AGPS 1:15	50 µl DRPS 1:20
	20 µl NGPS 1:1500	50 µl NRS 1:200

Control Plasma:

Adult pool	Pooled dove	Pooled male
(20.4 ng ml^{-1})	(8.3 ng ml^{-1})	(5.4 ng ml^{-1})

Inter-assay variation:

3.1%	4.0%	6.13%

Intra-assay variation:

0.76 - 2.8%	1.7%	4.6%

2.9.4 Data Reduction

'Bound' to 'Free' ratios were calculated by comparing the counts in 'blank' and 'zero standard' tubes. The amount of labelled hormone bound to the antibody in the absence of unlabelled hormone represented 100% binding. The amount of labelled hormone bound decreases as the amount of unlabelled hormone is increased forming a sigmoid curve. The standard curve was linearised using logit-log transformations and iterative least squares regression analysis. Unknown sample potency estimates and 95% confidence limits were based on the pooled residual variance of the standard curve and sample and calculated using a computer programme (Robard and Lewald, 1970).

3.0: THE ANATOMICAL BASIS OF THE PUTATIVE MONOAMINERGIC CONTROL OF ANTERIOR PITUITARY GLAND FUNCTION.

The original mapping of monoamine-containing neurone systems in the mammalian (Fuxe, 1965; Pin et al, 1968) and avian brain (Fuxe and Ljunggren, 1965; Sharp and Follett, 1968; Ikeda and Gotoh, 1971) made use of a formaldehyde-induced fluorescence (FIF) technique developed by Dahlstrom and Fuxe (1964). Modifications to the FIF technique (eg Bloom and Battenberg, 1976; Ajelis et al. 1979; Maeda et al. 1979), and their application to fresh frozen tissue (eg: Loren et al. 1980), have vastly improved the detection of catecholamines in brain tissue. Unfortunately the FIF and other histofluorescent methods have a low sensitivity towards 5-hydroxytryptamine (5HT), and the yellow fluorescent product formed (B-carboline) is highly sensitive to ultra-violet irradiation resulting in rapid fading of the fluorescence (Schofield and Wreford, 1979).

Alternative methods used to study central 5-HT containing neurones include autoradiography of radiolabelled 5HT taken up into the brain after intracisternal injection (Calas et al. 1974; Chan-Palay, 1977,1982) and immunohistochemical localisation of tryptophan hyroxylase and DOPA-decarboxylase (Hokfelt et al. 1973; Joh et al. 1975; Pickel et al. 1977). More recently direct localisation of 5-HT has been achieved by immunofluorescent techniques (Steinbusch and Nieuwenhuys 1981, 1983; Steinbusch, 1981; Howe et al. 1983) and peroxidase-antiperoxidase based immunohistochemistry (Steinbusch et al. 1982 a,b; Kojima et al. 1983) using antibodies raised in rabbit against 5HT conjugated to bovine serum albumin.

In the present study,the sucrose-phosphate-glyoxylic acid method was used to localise catecholaminergic neurone systems in the hypothalamus, and the avidin-biotin based immunohistochemical technique was used to demonstrate the 5HT-containing neurone systems in the brain.

3.1 Distribution of 5HT-Immunoreactivity in the Hind-brain and Hypothalamus

The perikarya of 5HT-immunoreactive neurones were mainly found in the hind-brain. A single grouping of perikarya was also found in the paraventricular organ of the hypothalamus. Fine fibres containing 5HT-immunoreactivity were found throughout the hypothalamus and anterior hind brain.

3.1.1 Distribution of 5HT-Immunoreactive Perikarya

Immunoreactive perikarya occurring in the hind brain displayed regional variations in size, number, and density of staining. Five cell groupings were discernible in the medial hind-brain (see fig 4).

Group 1: These cells, forming the most posterior grouping observed, appeared as weakly staining spindle shaped cells, sparsely distributed bilaterally medial to the nucleus nervi hypoglossi.

Group 2: These perikarya were large, multipolar and strongly immunoreactive, and were distributed within the middle portion of the raphe nucleus. Although forming a narrow band around the midline (see Fig 5A), they were extensively distributed rostro-caudally. The rostral end extended to the level of the nucleus nervi facialis.

Group 3: Immunoreactive perikarya in this group were of medium size and usually multipolar. They were widely distributed in the medial and paramedial portions of the middle pons (fig 5B).

Group 4: These cells were small and lightly stained with a lateral distribution in the ventral pons close to the lateral fasciculus longitudinalis medialis (Fig 5C).

Group 5: This large group of densely packed perikarya occurred mainly in the linea caudalis,ventral to the decussatio brachiorum conjunctivorum of the pons (Fig 5C).

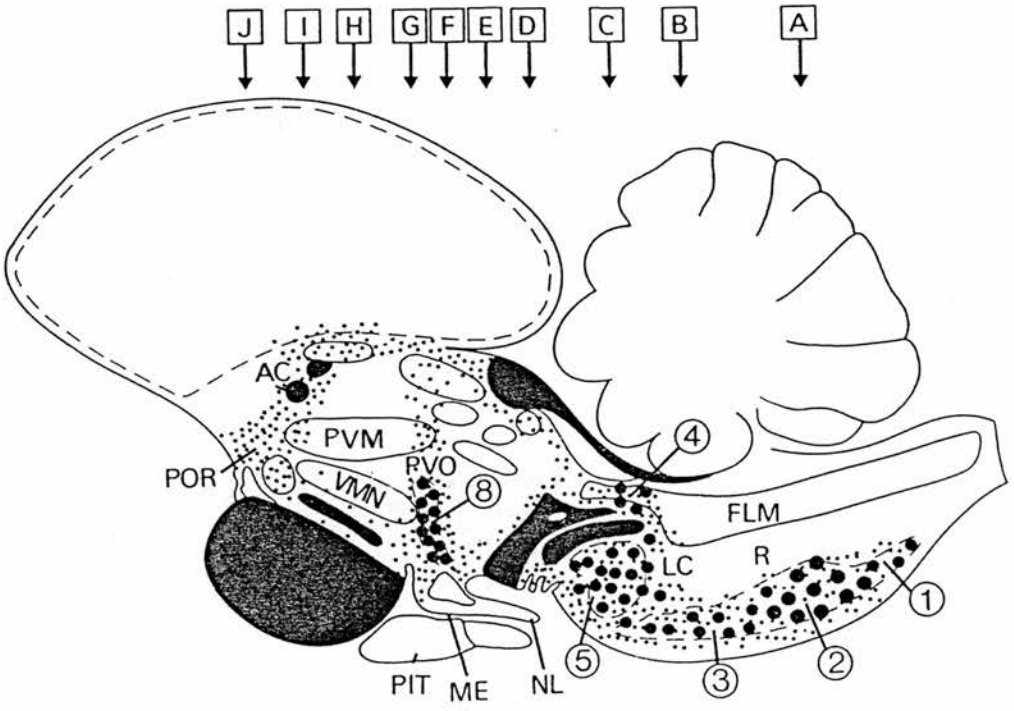
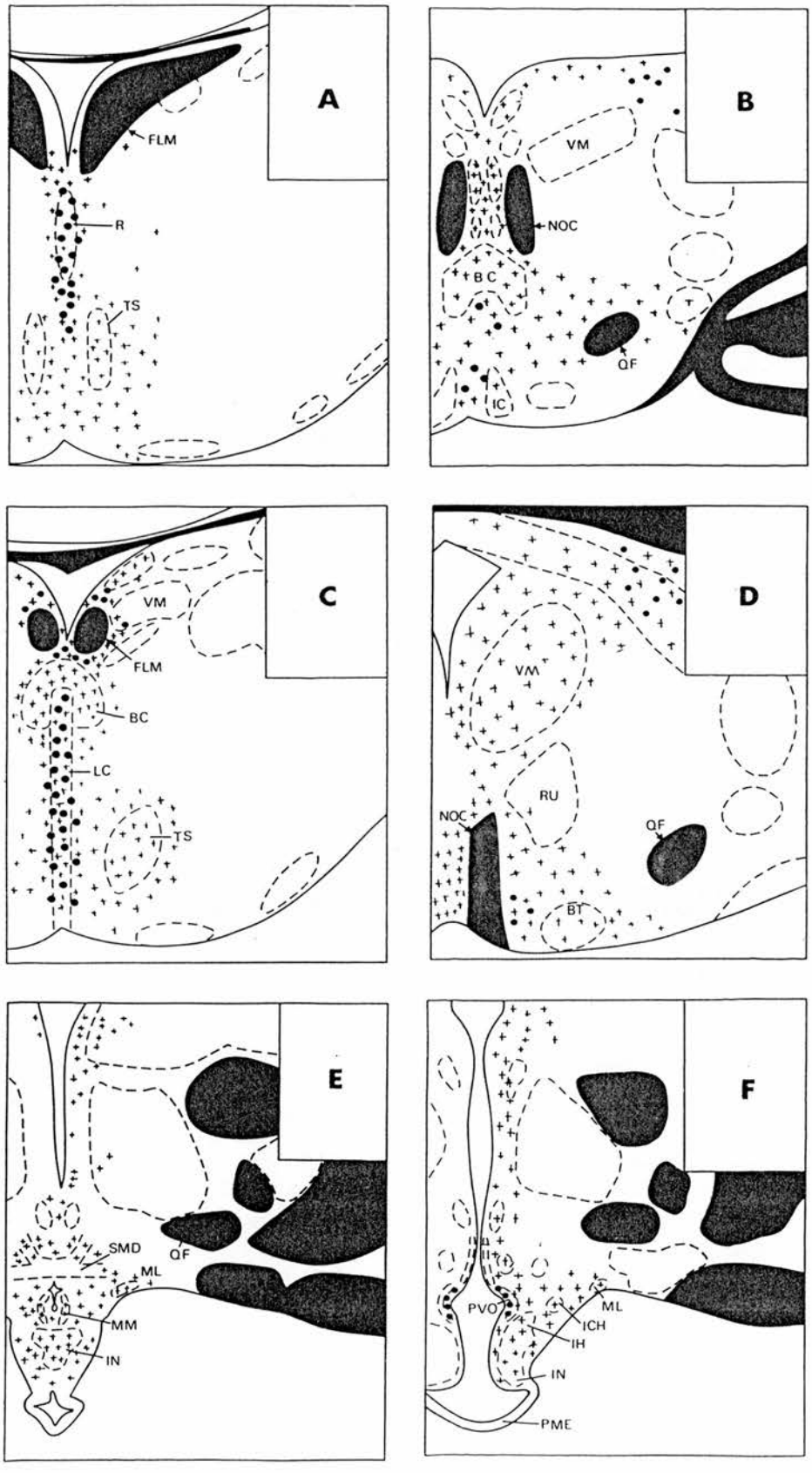


Figure 4: 5-hydroxytryptamine (5HT) immunoreactivity in the bantam brain. 5-HT was located immunohistochemically in para-sagittal preparations of 6 brains and plotted on a representation of the brain 0.5mm lateral to the midline. Perikarya containing 5HT are denoted by solid circles, while areas abundant in fibres and terminals containing 5HT are denoted by stipling. The groups of cells, numbered 1-8, are described in detail in the text. The letters A-J refer to the transverse representations of the bantam brain in figure 5. Abbreviations: AC anterior commisure, FLM fasciculus longitndinalis medialis, LC nucl. linearis caudalis, ME median eminence, NL neural lobe, PIT pituitary gland, POR pre-optic region, PVM nucl. paraventicularis magnocellularis, PVO para-ventricular organ, R nucl. raphe, VMN nucl. ventromedialis hypothalami.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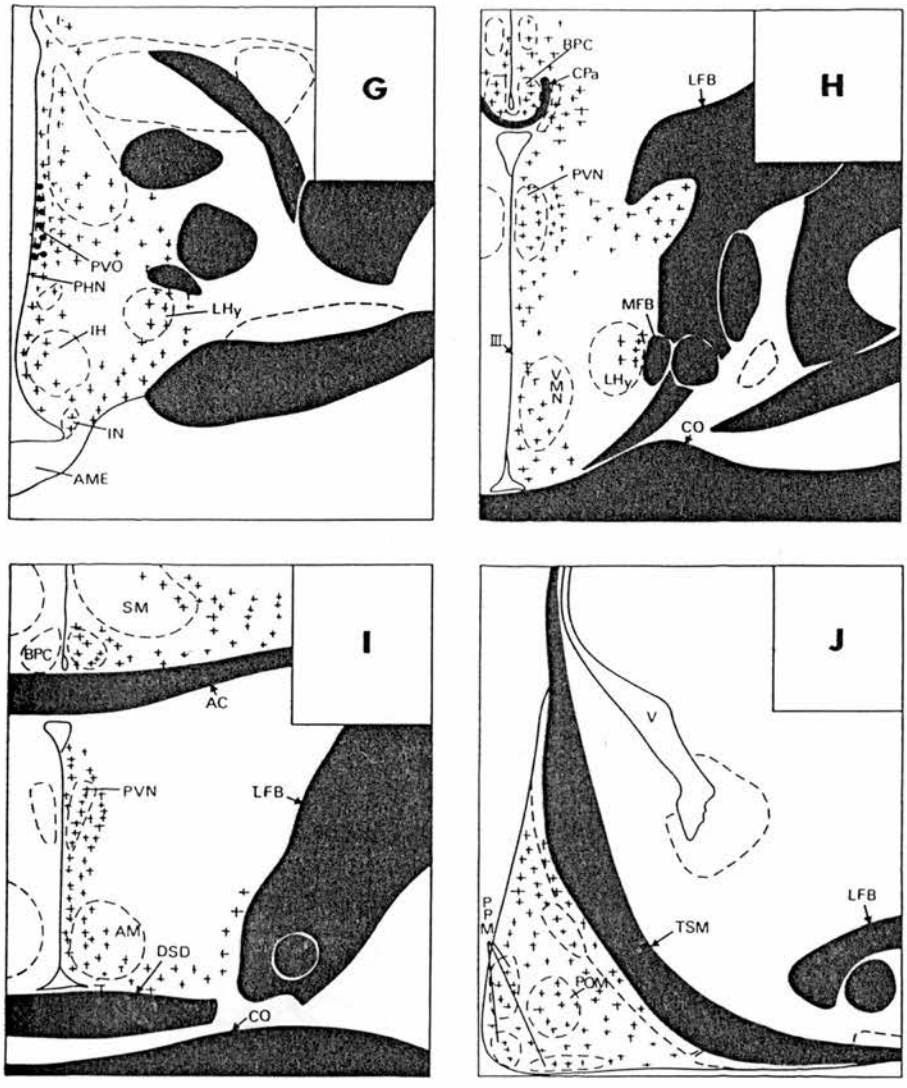


Figure 5: A-J Distribution of 5HT-immunoreactive perikarya (•) and fibres (+) in transverse sections through the bantam brain. The planes of sections are shown by the arrows in figure 4. These representations were compiled from observations of 5HT immunoreactivity detected in transverse sections of 11 brain preparations. Abbreviations; see appendix.

An additional two groups (6 and 7) of weakly staining 5HT-immunoreactive perikarya were observed in the lateral hind brain (Fig 5B and Fig 5D).

Within the hypothalamus, immunoreactive perikarya were observed in the paraventricular organ (group 8 in Figure 4). The cells formed a laminar structure around the third ventricle and were bipolar (Fig 5G + 5H). One process of each cell usually projected towards the wall of the ventricle and may be similar to the 'liquor-contacting' neurones described by Vigh-Teichman et al (1970). The other process extended laterally towards the infundibular nuclear complex.

This distribution of perikarya is consistent with the observations of Yamada et al. (1984) in the young chicken.

3.1.2 5-HT Immunoreactive Fibres

Fine immunoreactive fibres and characteristically punctate terminals occur throughout the hypothalamus and hind brain. Relatively coarse, beaded fibres were found only in regions adjacent to immunoreactive cells. Although apparently ubiquitous in their distribution, areas which contained higher densities of fine, immunoreactive fibres were discernible.

A major division of fibres originating from cells in the hind brain occurred at the level of the oculomotor nerve (Fig 5D + 5E); one group passed superior to the fasciculus longitudinalis medialis, passing through the upper part of the posterior hypothalamus and spreading throughout the pre-optic area and septal region (~~Fig 5K~~), while the other group passed laterally round the oculomotor nerves into the basal hypothalamus (Fig 5E), towards the lateral tuberal area. Coarse beaded fibres emanating from the cells in the paraventricular organ passed laterally and descended through the lateral basal hypothalamus towards the infundibular nuclear complex (Fig 5G + 5H). Dense fibre staining occurred in the lateral tuberal area extending downwards to the lateral margins of the median eminence (Fig 5F + 5L + 5H). Dense fibre and terminal staining also occurred in the anterior hypothalamus. Large numbers of fine immunoreactive fibres were found around the region of

the anterior commissure (Fig 5I). The pre-optic region itself was rich in immunoreactive fibres and terminals which were not associated with particular nuclear groupings (Fig 5J). Fine terminal staining was also found in the organosum vasculosum lamina terminalis. A few fibres with a rostro-caudal orientation were observed above the supraoptic decussation between the preoptic area and the basal hypothalamus (Fig 5I + 5J)

3.2 Catecholamine Distribution in the Hypothalamus

Studies in the quail (Sharp & Follett, 1968) and chicken (Ikeda and Gotoh, 1971; Duabe and Parent, 1981), have outlined the distribution of catecholamine containing structures in the avian brain. A limited study was conducted to confirm the hypothalamic distribution of the catecholamines in the hypothalamus of the bantam hen.

When exposed to ultra-violet light after immersion in a solution containing glyoxylic acid (de la Torre and Surgeon, 1976) cryostat sections of fresh frozen tissue emitted yellow and green fluorescence . The yellow fluorescence associated with 5-HT faded quickly when examined, while the green fluorescence associated with the catecholamines persisted. No detailed examination of the hind brain was conducted but subjectively the monoamine perikarya observed in this region corresponded to the groupings outlined by Ikeda and Gotoh (1971). In the hypothalamus strong green fluorescence occurred in cells of the paraventricular organ. Beaded fibres radiating from these cells were observed to descend laterally towards the infundibular region. Heavy catecholamine fluorescence occurred in the posterior tuberal area associated with the nucleus mammilaris lateralis and the infundibular nuclear complex. The lateral median eminence contained considerable amounts of catecholamine fluorescence and the internal layer of the median eminence contained fine catecholamine containing nerve fibres. Some fibres were seen to descend from the internal to the external layer of the anterior median eminence. The pre-optic region of the hypothalamus also contained abundant catecholamine fibres. No particular nuclear associations were observed in this region although catecholamine fibres were particularly dense in the medial and ventral

regions of the nucleus praeopticus paraventricularis magnocellularis. The catecholaminergic innervation of the anterior hypothalamus was similar to that observed for 5HT. Figure 7 contains photomicrographs of the catecholamine fluorescence.

3.3 Discussion: Possible Sites at Which Monoamines May Influence Neuroendocrine Function

The current understanding of the avian hypothalamic neurosecretory system is based on anatomical evidence suggesting the organisation of neurosecretory cells into two distinct components. These are the classical aldehyde-fuchsin positive magnocellular neurosecretory system and the aldehyde-fuchsin negative parvocellular or hypophysiotrophic system (Mikami, 1980). The median eminence and neurohypophysis are the common anatomical end points of these convergent systems. The magnocellular neurosecretory cells are thinly dispersed throughout the preoptic-hypothalamic transitional areas in the rostral hypothalamus and send efferent fibres to the median eminence and neurohypophysis via the hypothalamo-hypophyseal tract. The parvocellular cells located in the basal and anterior hypothalamus send efferent fibres only to the median eminence (Mikami et al. 1978)forming the tubero-hypophyseal tract.

The identification and analysis of individual neuronal systems in the avian brain by means of histochemistry and more recently immunohistochemistry, have demonstrated the complex heterogeneous nature of the avian hypothalamic nuclei (Oksche, 1983).

Several neuropeptide systems have been identified immunohistochemically in the chicken central nervous system. Both neurohormonal and/or neurotransmitter roles have been ascribed to the various neuropeptides on the basis of their anatomical relationship to other neurones and neurohaemal areas in the brain (Blahser, 1983). Of the petidergic systems known to have a neurohormonal function, the LHRH-system has particular relevance to the endocrine changes associated with broodiness. The distribution of the LHRH-system in the chicken brain has been described by Sterling and Sharp (1982). The LHRH-containing cell bodies are located in the rostral anterior hypothalamus, in front

of the classical magnocellular neurosecretory cells,and project fibres through the ventral hypothalamus which terminate in the median eminence.

The similarities in anatomical location within the hypothalamus between monoaminergic neurones and elements of the avian neuropeptidergic system, suggest that interaction between the two systems may occur at several levels:

1. Within the median eminence, a dense catecholaminergic innervation was observed, and although little 5-HT immunoreactivity could be detected , measurements of this monoamine, using HPLC with electrochemical detection, show it to be present in high concentrations (Talbot et al. 1984). Monoamines in the median eminence may influence hormone secretion from the pituitary gland, by an interaction with neuropeptidergic terminals to regulate the release of peptide factors into the hypophyseal portal vasculature. In addition they may also modulate the effects of neurohormonal releasing/inhibiting factors at the level of the target cell in the pituitary gland.

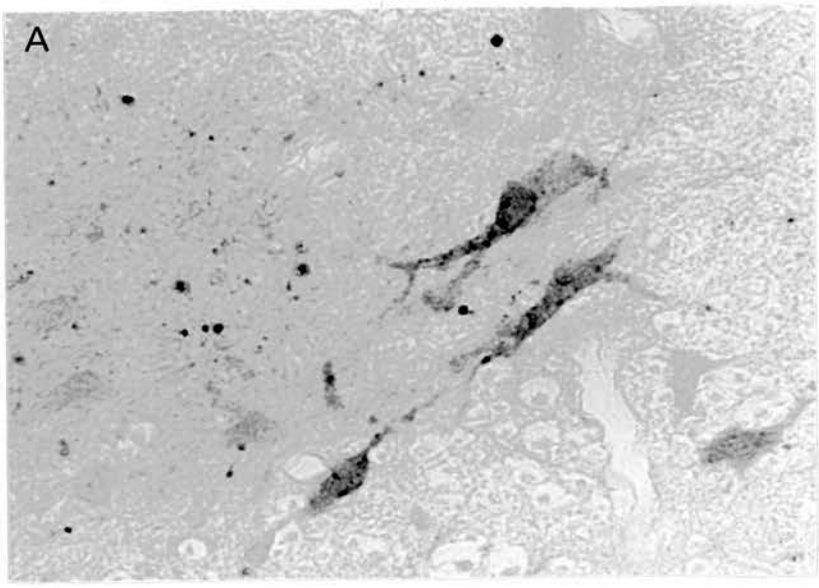
2. The activity of the hypophysiotrophic neurosecretory cells in the basal hypothalamus may be modulated by the monoamine - containing terminals which originate in the hind brain and the paraventricular organ.

3. The intense innervation of the anterior hypothalamus by monoaminergic systems may influence the activity of the magnocellular neurosecretory cells and LHRH-containing cells.

4. Further, more complex, interactions between monoaminergic and neurosecretory neurones may also occur if these peptide systems are ascribed neurotransmitter/neuromodulatory roles.

The anatomical proximity of elements of the neurosecretory cells and 5-HT and catecholaminergic systems although suggestive of an interaction, is not adequate evidence for a functional association.

Only the presence of synaptic connections between monoaminergic and peptidergic systems, which will require detailed immunohistochemical investigation, at the electron microscopic level, will confirm these possible links. However, indirect evidence of an interaction may be found by a detailed study of the functional activity of monoaminergic systems in various endocrine states, and the effects on hormone secretion of drug treatments which affect specific components of monoaminergic function.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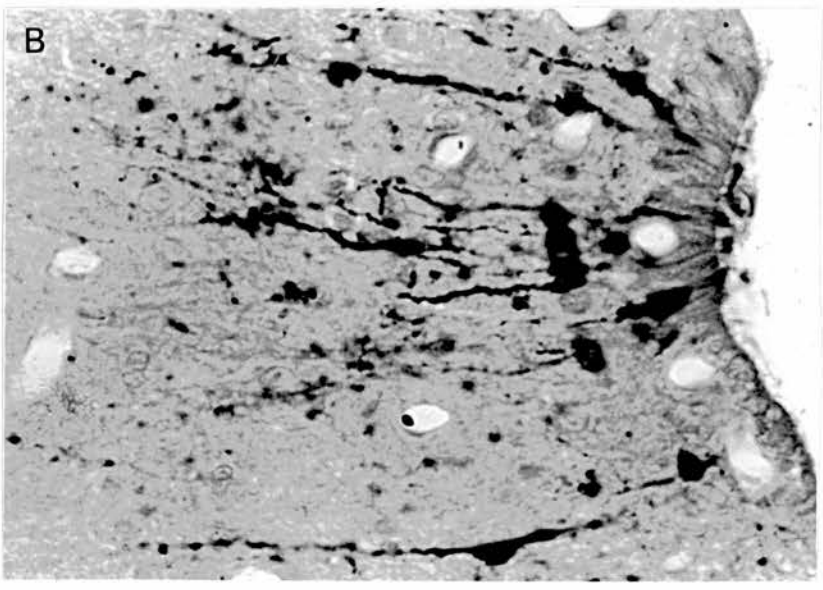
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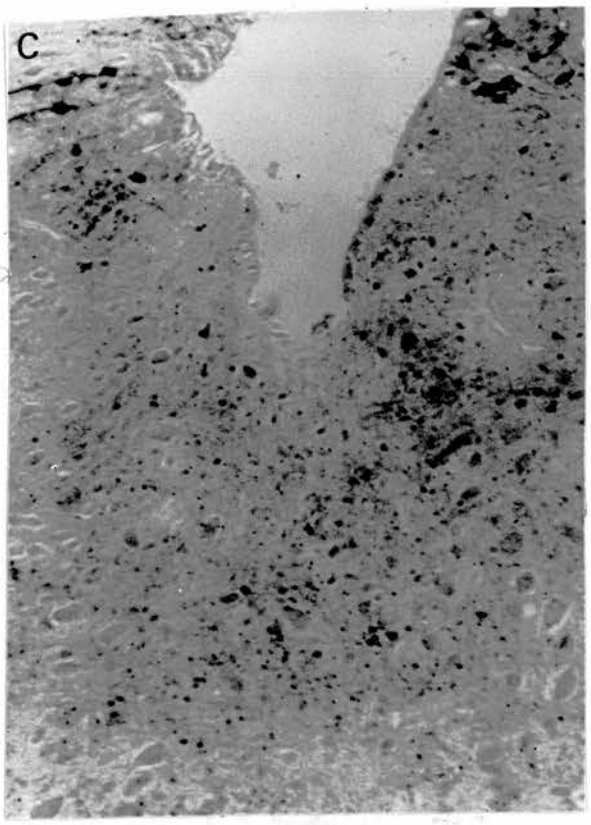


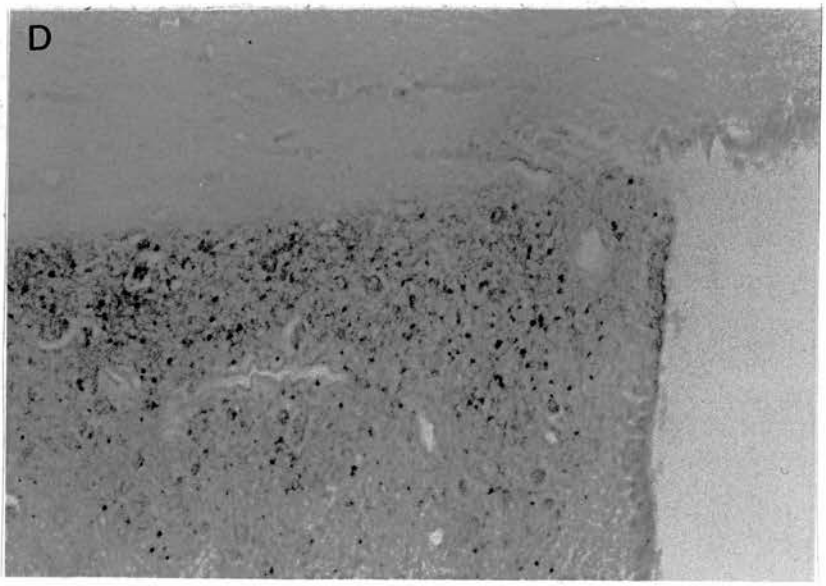


Figure 6: Photomicrographs of 5HT-immunoreactivity in the bantam brain;
A. Perikarya in the dorsal raphe nucleus
B. Perikarya in the para-ventricular organ
C. Fibres in the region of the infundibular nuclear complex
D. Fibres below the anterior commissure

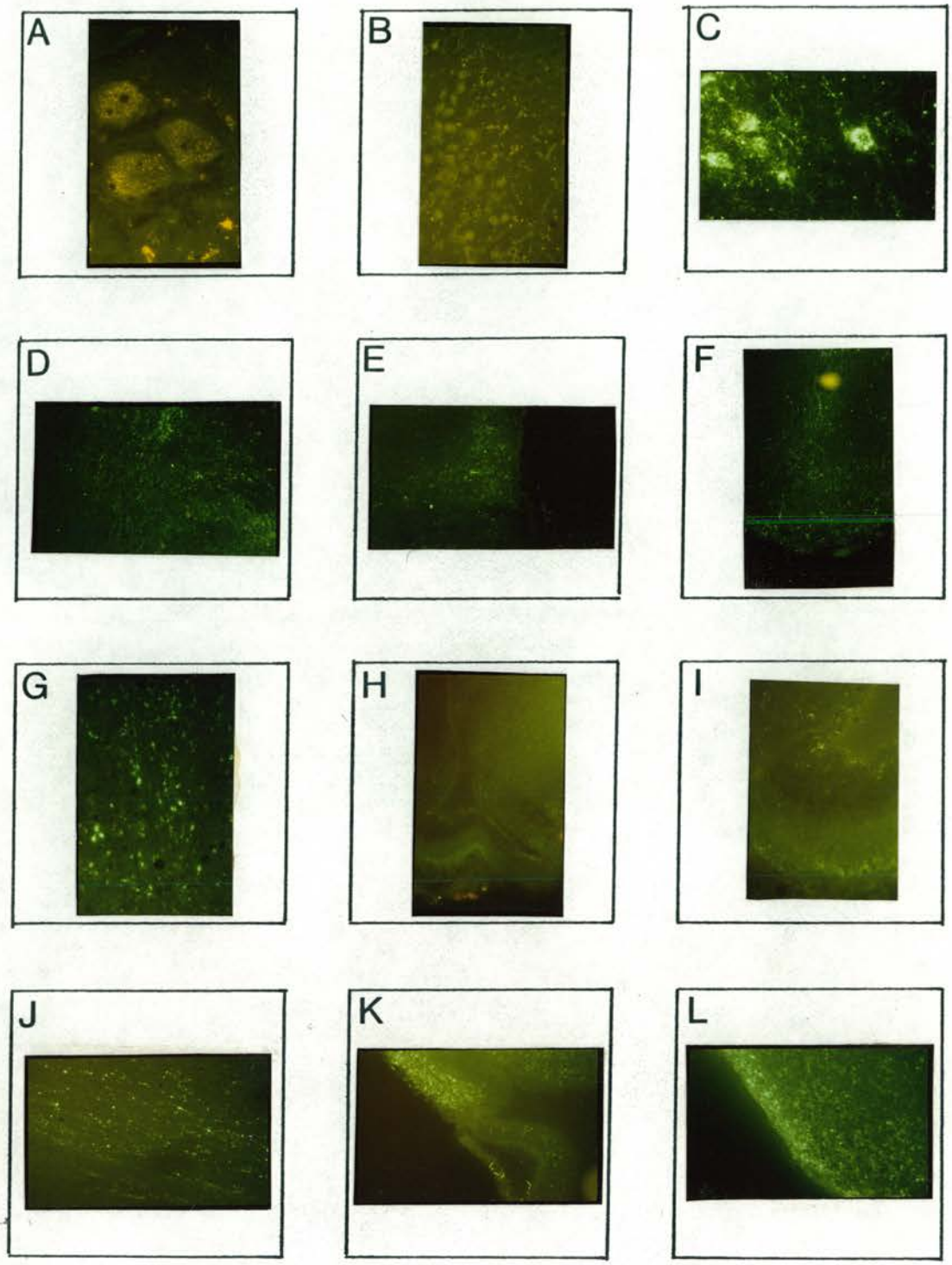


Figure 7: Photomicrographs of mono-aminergic fluorescence in the bantam brain; A:Cells in the dorsal raphe nucleus (x40). B:Cells in the nucl. linearis caudalis (x40). C:Cells in the PVO (x40). D:Fibres in the region of the NOC (x10). E. Fibres in the lateral tuberal area (x10). F. Fibres in the Infundibular Nuclear Complex (x10). G. Fibres in the INC (x40). H. Sagittal section through the median eminence (x20). I. Sagittal section through the median eminence (x40). J. Fibres running rostrocaudally in the region of the Supra-optic decussation (x20). K.Fibres and terminals in the OVLT (x20). L. Fibres in the POA, sagittal section (x10).

4.0: THE FUNCTIONAL ACTIVITY OF HYPOTHALAMIC MONOAMINES IN BROODINESS

The onset of incubation in the bantam hen is characterised by an increase in the plasma concentration of prolactin and a decrease in the plasma concentration of luteinizing hormone (Lea et al. 1981). After the eggs hatch, plasma levels of prolactin fall, while those of luteinizing hormone (LH) progressively increase (Sharp et al. 1979). In the turkey, a similar fall in plasma prolactin (El Halawani et al. 1983) accompanied by an increase in plasma LH (EL Halawani et al. 1983) occurs if incubation is terminated prematurely by removing the nest. Since the secretion of LH and prolactin in turkeys and chickens is probably controlled, at least in part, by monoamines (see section 1.2 and 1.3) it is likely that hypothalamic monoamines are involved in the regulation of LH and prolactin secretion in broody bantams. The abundant innervation of the bantam hypothalamus with neurones containing 5-hydroxytryptamine (5HT) and catecholamines ,provides the anatomical basis for such a regulatory mechanism.

The primary purpose of the following series of experiments was to correlate the changes in the concentration and turnover of monoamine neurotransmitters in the hypothalamus, with the changes in the secretion of prolactin and LH associated with the development and termination of incubation. Since incubation can be conveniently terminated by removal of the nest ,an initial study was performed to determine the effects of nest removal on plasma levels of prolactin and LH. It was anticipated that the nest-deprived bantam hen would provide a convenient model for studying changes in hypothalamic function immediately after reversing the changes in prolactin and LH secretion which occur at the onset of incubation.

4.1 The Effect of Nest-Deprivation on Plasma Levels of Prolactin and LH

The effect on plasma levels of prolactin and LH of depriving incubating hens of their nests for 24 hours, is shown in figure 8. Shortly after nest removal,prolactin levels began to fall and after 12 hours were significantly lower than in the non-deprived control group ($p < 0.01$, unpaired t-test, n=6). All the nest-deprived hens re-started incubating within 10 minutes of being returned to their nests. Re-nesting was accompanied by a rapid rise in plasma prolactin. Plasma levels of

prolactin were not significantly different from those in control birds 12 hours after nest return. A similar pattern of change in plasma levels of prolactin was detected when the same samples were assayed in the heterologous prolactin radioimmunoassay.

Within 3 hours of nest-removal, plasma levels of LH were significantly increased when compared with control values ($p < 0.01$, unpaired t-test), and remained elevated until the birds were allowed to re-nest. After re-nesting plasma levels of LH fell rapidly to those of control birds which had nested throughout the experiment.

Depriving incubating hens of their nests caused an acute reversal of prolactin and LH secretion resulting in a plasma prolactin and LH profile similar to that found in laying birds. However, nest-deprived hens maintain the drive to incubate, which was evident by the acute reversal of prolaction and LH secretion which occurs after return to the nest.

4.2 'Steady-state' Concentrations of Hypothalamic Monoamines

Figure 9 shows the concentrations of monoamines in the anterior and posterior hypothalami of laying, incubating,and nest-deprived-incubating (NDI) hens.

The concentration of 5-hydroxytryptamine (5HT) in the anterior hypothalamus was higher in NDI than laying birds and in the posterior hypothalamus was higher in incubating than NDI birds . Apart from these differences the concentrations of the individual monoamines did not vary with endocrine state within either division of the hypothalamus. The only difference in monoamine content between the two parts of the hypothalamus was for noradrenaline (NE) which was present at a higher concentration in the anterior hypothalamus when compared with that in the posterior hypothalamus of the equivalent groups($p<0.05$, unpaired t-tests, $n=8$). Any attempt to ascribe physiological significance to these differences must assume that changes in the concentration of a particular monoamine, reflects a change in the functional activity of the neurone system in which that amine functions as a neurotransmitter. The lack of a consistent relationship between amine concentration and

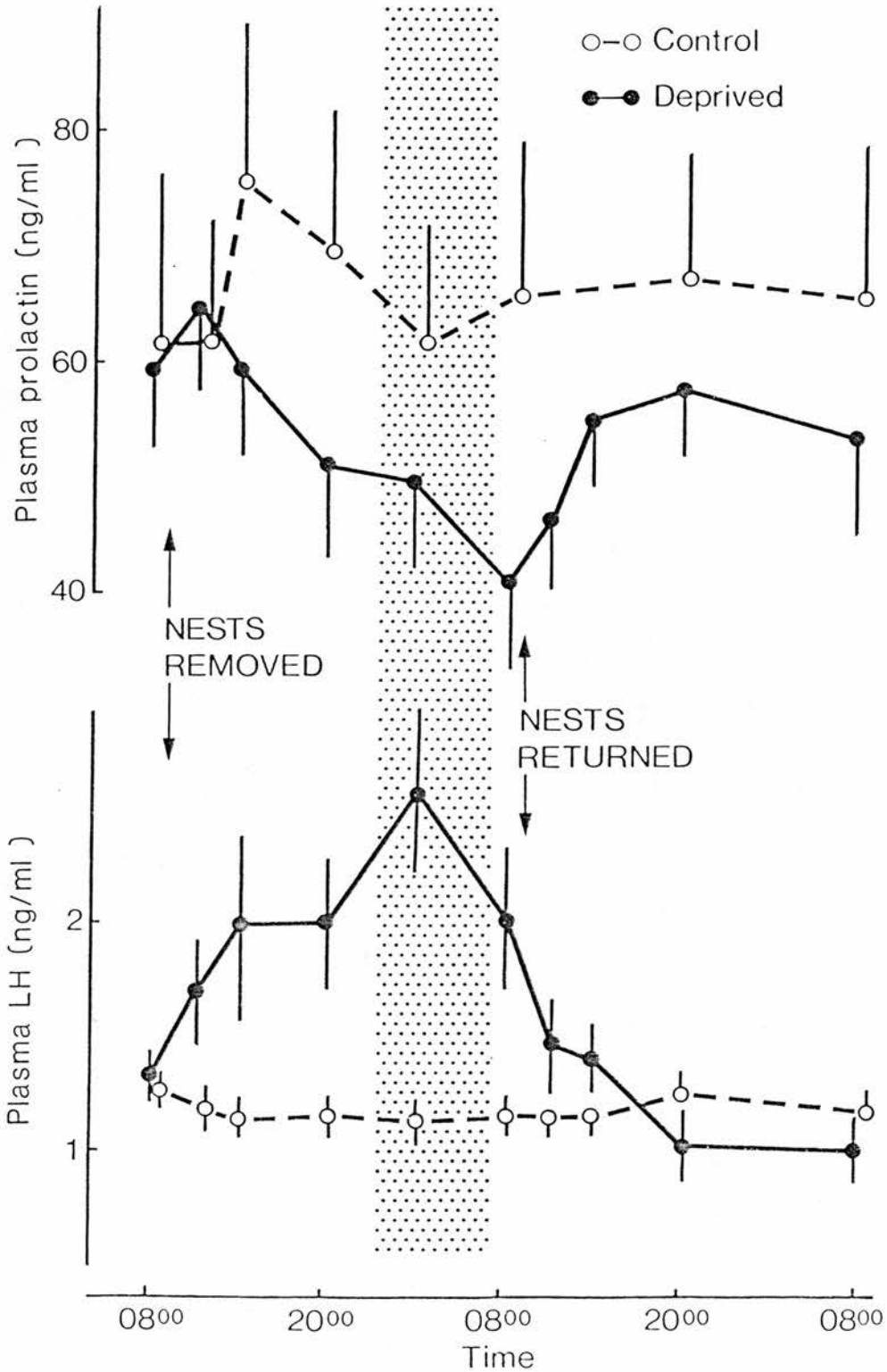


Figure 8: The effect of a 24 hour nest deprivation on plasma levels of prolactin and luteinizing hormone (n = 6, ∓ SEM). Serial blood samples were taken from bantam hens deprived of their nests for 24 hours (●–●) or allowed to continue incubation (o--o). Plasma samples were measured for prolactin and LH in homologous radioimmunoassays. Selected samples assayed for prolactin in the heterologous radioimmunoassay showed a similar pattern of change (data not presented).

The stippled area represents the 'dark' period.

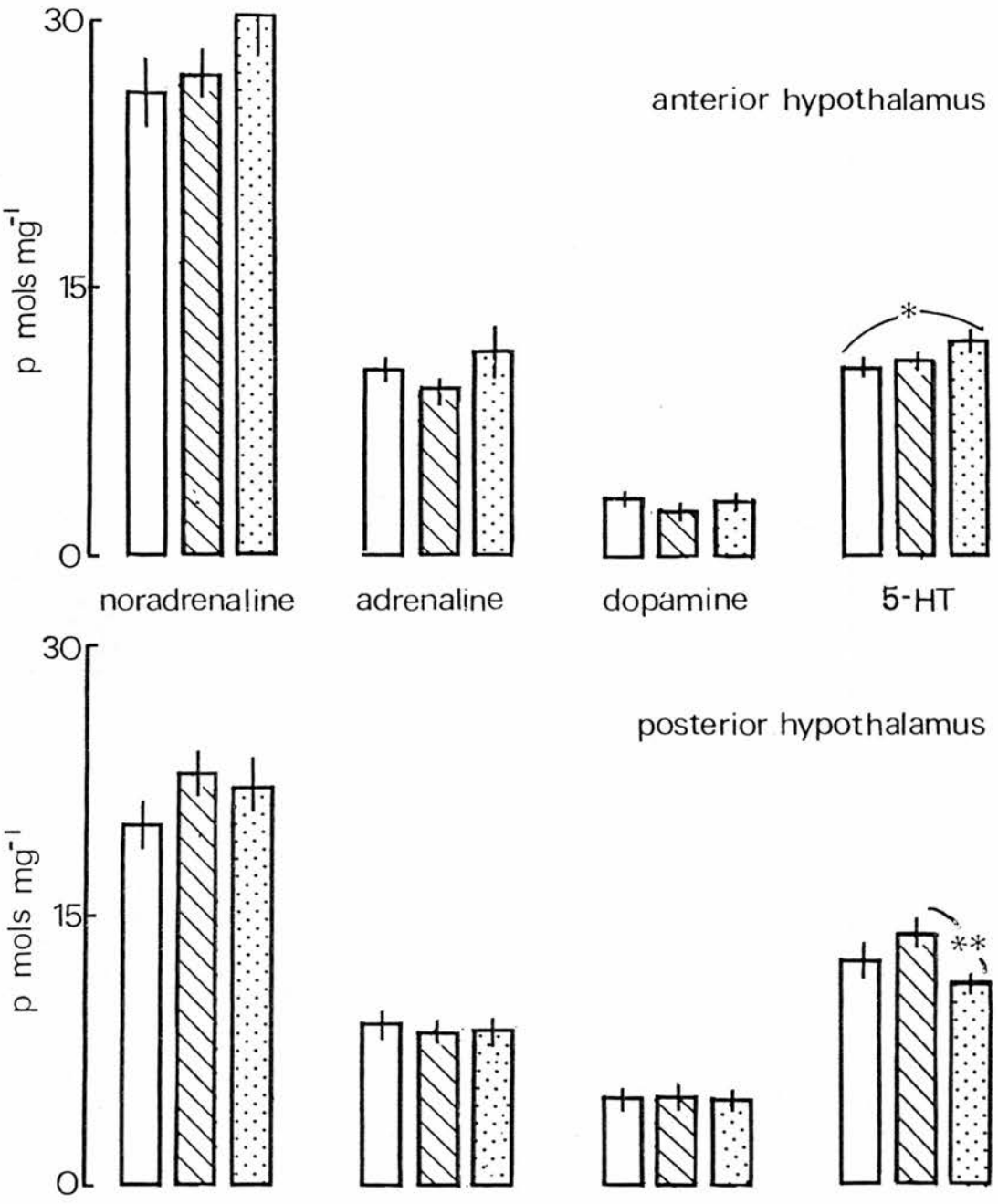


Figure 9: Monoamine neurotransmitter concentrations in the hypothalamus. Monoamine concentrations in the anterior hypothalamus and the posterior hypothalamus of laying (□), incubating (▨) and incubating bantam hens which had been deprived of their nests for 24 hours (▩) were determined by electrochemical detection after HPLC. Results are expressed as the mean ± S.E.M. of 8 observations (**-$p<0.01$, *-$p<0.05$, unpaired t-test).

turnover rate in the mammalian hypothalamus (eg. Crowley et al. 1978; Moyer et al. 1979), questions the validity of this assumption. Therefore the functional activity of a monoaminergic neurone system is better assessed as the turnover rate of its monoamine transmitter. In the present study an index of turnover, which was thought to be proportional to turnover rate, was used.

4.3 Estimation of Functional Activity

Several non-isotopic methods have been employed to estimate monoamine turnover rate. In the domestic fowl, the ratio of major metabolite to parent amine has been used (Juorio and Vogt, 1967). This approach is limited by the low levels of acid metabolites found in chicken brain which reflect the presence of a highly effective transport system for acid metabolites in the avian brain (Ahtee et al. 1970). The levels of acid metabolites in brain tissue can be enhanced by the administration of probenecid which blocks the active transport system (Tozer et al. 1966). However, probenecid has been shown to be sedative in chickens at the dose required to block acid metabolite excretion (Ahtee et al. 1970).While allowing a better assessment of these metabolites, such a side effect may alter turnover rate. Turnover rate has been estimated in turkey brain by measuring the decrease in monoamine concentration after the inhibition of synthesis (El Halawani et al. 1980) and by measuring the increase in monoamine concentration after the inhibition of catabolism (El Halawani and Burke. 1976). However, blockade of amine synthesis or catabolism may cause adaptive changes in the functional activity of the monoaminergic neurone (von Euler, 1972; Burnstock and Costa, 1975).

In the present study, two methods were used to estimate turnover. Firstly,the ratio of monoamine to metabolite was used to estimate the turnover of 5-hydroxytryptamine (5HT) and dopamine (DA). Secondly, the accumulation of amine after the inhibition of the catabolic enzyme, monoamine oxidase with pargyline was used to estimate the turnover of noradrenaline (NE), adrenaline (A), 5-HT and DA. Although previous

reports (Tozer et al. 1966; Ahtee et al. 1970) suggest that pargyline acts as a mild sedative, no sedation was observed in treated birds at the dose used in this study.

4.3.1 The Ratio of 5-hydroxyindole Acetic Acid (5HIAA) to 5-hydroxytryptamine (5HT)

The concentrations of 5HT and its major metabolite 5HIAA were estimated in samples of brain tissue by HPLC with electrochemical detection. The ratio of 5HIAA to 5HT was used as an indicator of the relative functional activity of 5HT neurones in the hypothalami of laying, incubating and nest-deprived-incubating (NDI) bantam hens (Fig 10). The 5HIAA/5HT ratio in the anterior hypothalamus was highest in incubating birds and similar in laying and NDI groups. In the posterior hypothalamus the ratio was highest in the NDI group while laying and incubating values were similar. By implication, the patterns of 5HT turnover in the anterior and posterior hypothalamus were strikingly different within each group of birds; in laying birds a higher turnover of 5HT was found in the posterior hypothalamus than in the anterior hypothalamus ($p<0.01$, unpaired t-test, $n=8$). In incubating birds, 5HT turnover was much higher in the anterior than posterior hypothalamus ($p<0.001$, unpaired t-test $n=8$), while in NDI birds 5HT turnover was higher in the posterior hypothalamus than the anterior hypothalamus ($p<0.001$ unpaired t-test $n=8$).

4.3.2 The ratio of Homovanillic Acid (HVA) to dopamine (DA)

Simultaneous determination of DA and its major metabolite, HVA, in samples of brain tissue, allowed the calculation of a ratio indicative of DA turnover rate (Fig 11). In the anterior hypothalamus the ratio of DA to HVA was higher in incubating birds than in laying or NDI birds ($p<0.01$ unpaired t-tests, $n=8$) and tended to be higher in laying than in NDI birds. There was no detectable difference in the DA:HVA ratio in the posterior hypothalami between the three groups. Within each group the DA:HVA ratio in laying and incubating birds was higher in the

anterior hypothalamus than in the posterior hypothalamus ($p < 0.05$, unpaired t-tests, n=8). No difference was detected between the anterior and posterior hypothalamus in NDI birds.

It was not possible to assess the turnover of NE or A by this method, due to difficulty in detecting the very small amounts of methoxy-3-hydroxyphenylglycol(MHPG) present in the samples and inconsistency in its retention time after HPLC.

4.3.3 Monoamine Accumulation After Monoamine Oxidase Inhibition

A pilot study was done to determine the rate of amine accumulation in the chicken hypothalamus after treatment with pargyline. Groups of pargyline treated birds were killed 0.75, 1.5 and 2.25 hours after injection, and control groups were killed at time 0 and 2.25 hours after a saline injection. The concentration of hypothalamic amines were measured and correlation coefficents and F scores were calculated after linear regression analysis of the resulting data (see Fig 12). Linear accumulations of hypothalamic 5HT, DA, NE and A were apparent in pargyline treated birds for 1.5h while no change in monoamine concentration occurred in control birds. This finding suggested that no adaptive changes in monoaminergic neurones occurred in the hypothalmus as a result of increased amine content after pargyline treatment over the time period studied. Table 1 summarises the results.

Turnover indices based on the rate of accumulation of the monoamines following pargyline treatment in the anterior and posterior hypothalamus of laying, incubating and nest-deprived incubating (NDI) birds are presented in figure 13.

In the anterior hypothalamus the turnover of noradrenaline (NE) was similar in laying and incubating birds but was not detectable in NDI birds. The turnover of NE was similar in the posterior hypothalamus in all groups of birds. In the anterior hypothalamus, the turnover of adrenaline was significantly higher in incubating than in laying birds, but was not detectable in hypothalami from NDI birds. Adrenaline turnover was significantly higher in the posterior hypothalami of

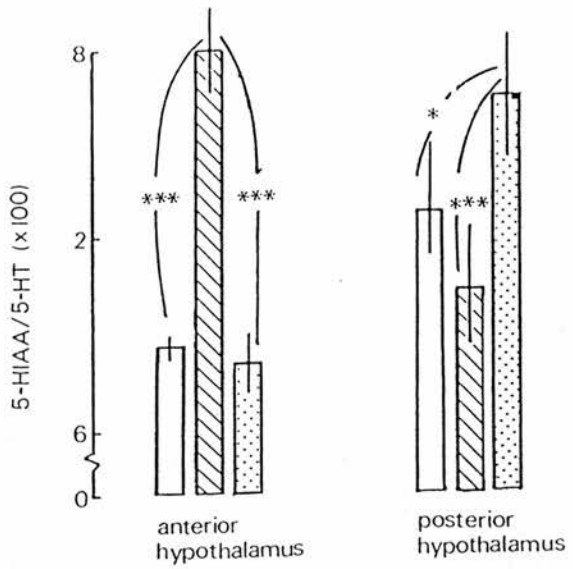


Figure 10: The ratio of 5-hydroxyindole acetic acid (5HIAA) to 5-hydroxytryptamine (5HT) in the anterior and posterior hypothalami of laying (□), incubating (▨) and incubating bantam hens which had been deprived of their nests for 24 hours (▩). The results are the mean ± S.E.M. of 8 determinations (***-$p<0.001$, *-p 0.05, unpaired t-test)

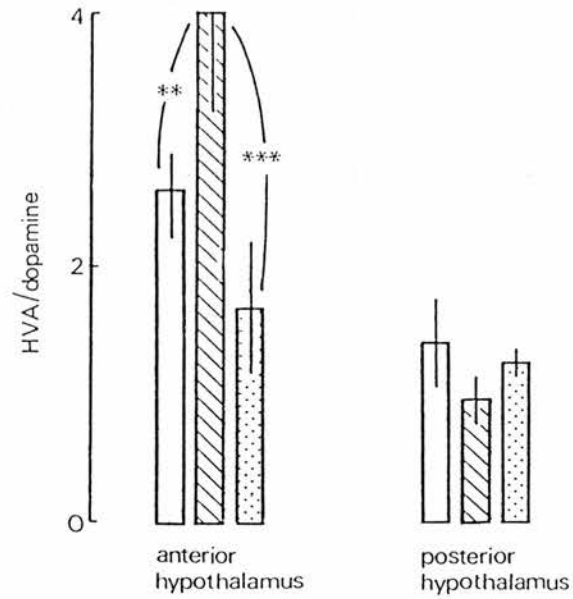


Figure 11: The ratio of homovanillic acid (HVA) to dopamine (DA) in the anterior and posterior hypothalami of laying (□), incubating (▨) and incubating bantam hens which had been deprived of their nests for 24 hours (▩). The results are the mean ± S.E.M. of 8 determinations (***-$p<0.001$, **-$p<0.01$, unpaired t-test)

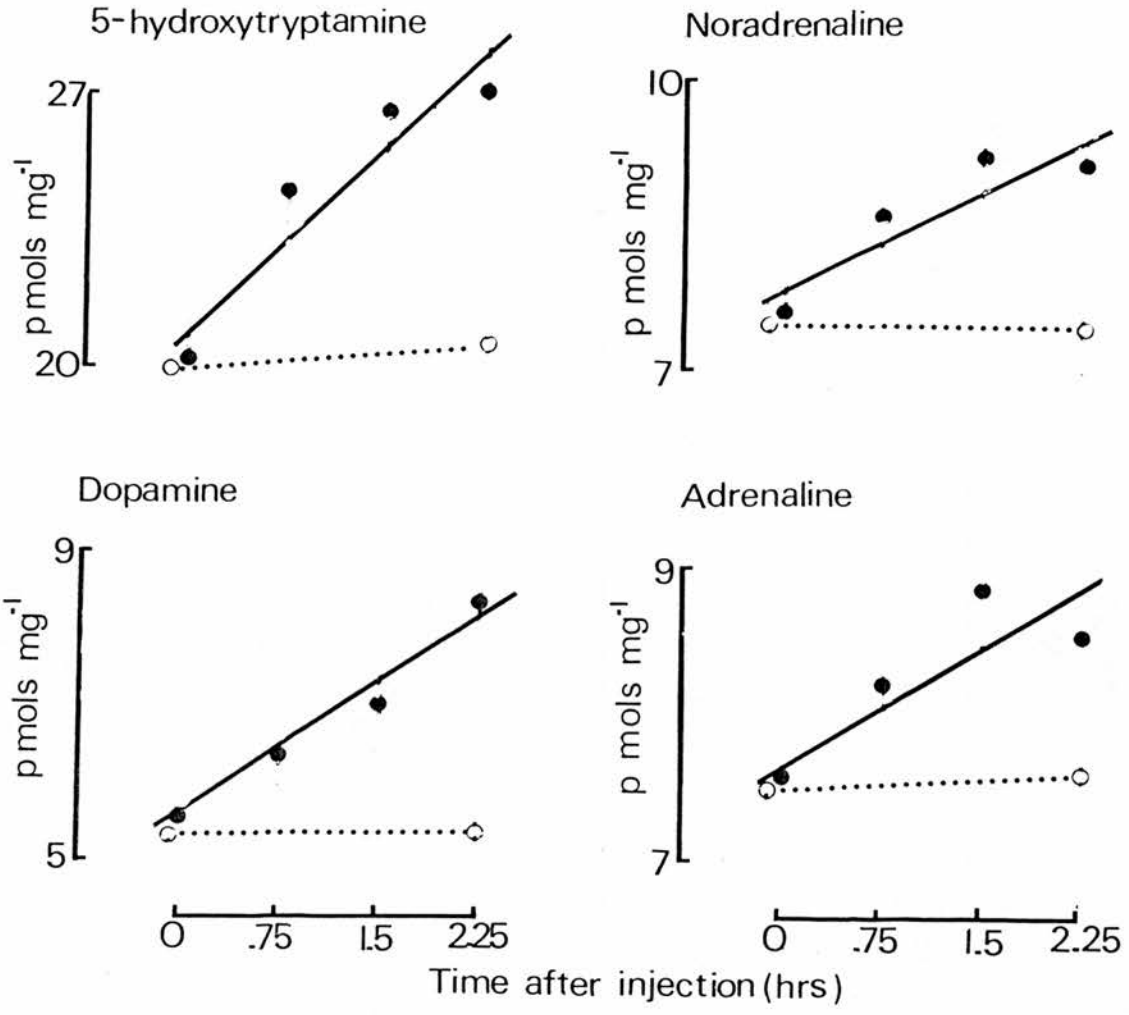


Figure 12: Monoamine accumulation after pargyline injection. The concentration of hypothalamic monoamine neurotransmitters were determined in groups of laying hens at intervals following an intraperitoneal injection of 75 mg/kg pargyline (●) or an equivalent volume of 0.9% saline (O·····O). Results are the mean of eight observations per time point. See table 1 for statistical analysis.

Table 1: Monoamine accumulation after pargyline treatment. These are the results of linear regression analysis of the data presented in Figure 12, and are expressed as the correlation coefficient (r), F-score (F) and minimum level of significance (df.=23).

	r	F	p
5-hydroxytryptamine	0.54	9.2	0.01
dopamine	0.66	18.1	0.001
noradrenaline	0.42	3.7	0.05
adrenaline	0.42	3.6	0.05

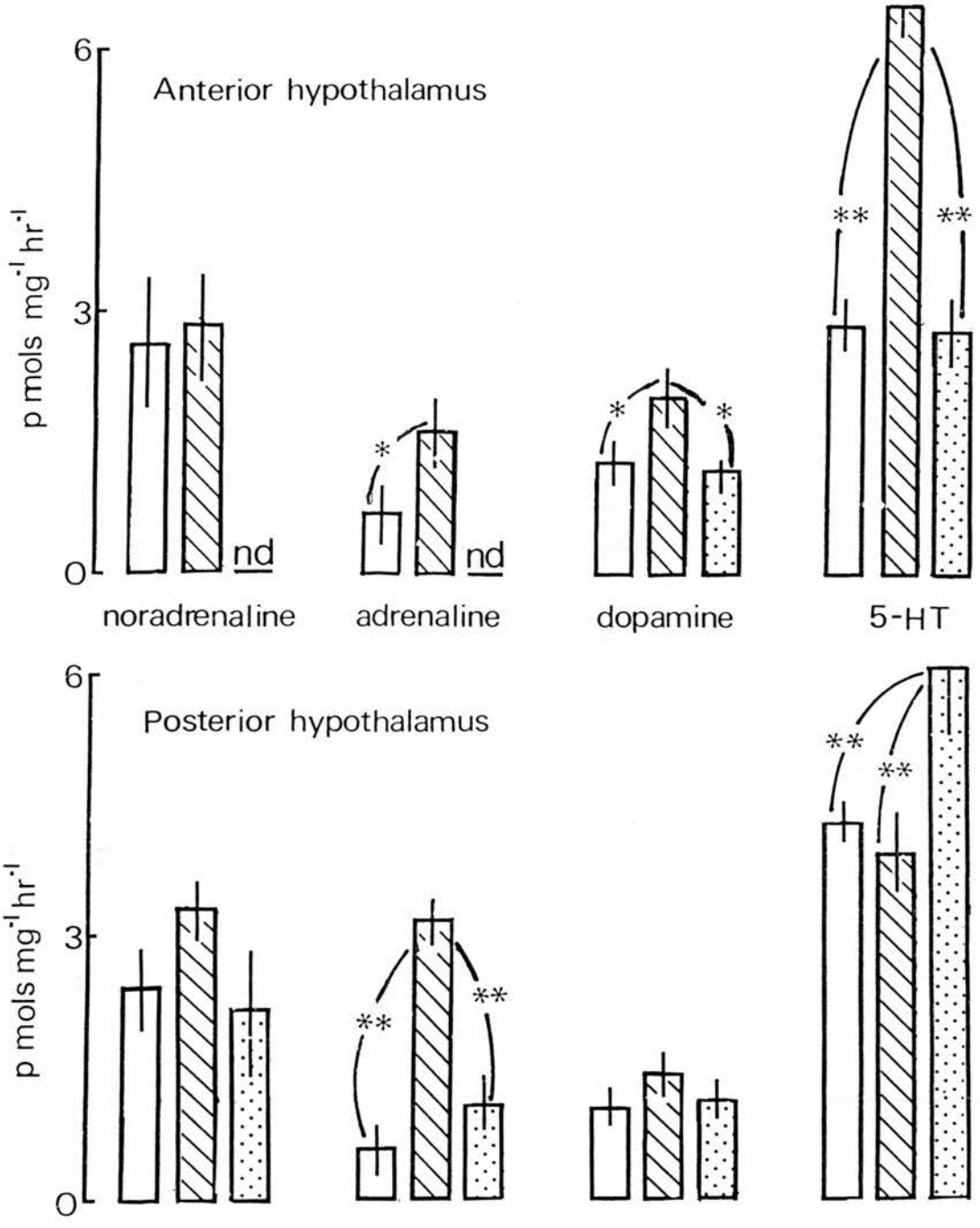


Figure 13: Indices of monoamine neurotransmitter turnover in the anterior and posterior hypothami of laying (□), incubating (▨) and incubating bantam hens which had been deprived of their nests for 24 hours (▩). The index of turnover was calculated by subtraction of the monoamine concentration in hypothalami from saline treated birds (n=8) from the monoamine concentration in hypothalami from pargyline treated birds (n=8) killed 2 hours after injection. (***-$p<0.001$, *-$p<0.05$, unpaired t-test, nd = non-detectable).

incubating birds than in laying or NDI birds, which had a similar turnover. Dopamine turnover in posterior hypothalami was similar in all three groups. In the anterior hypothalami dopamine turnover was higher in incubating than in laying or NDI birds. The most dramatic difference in turnover rates between the groups was for 5-hydroxytryptamine (5HT). The turnover of 5HT in the anterior hypothalamus was much higher in incubating than laying or NDI birds, while in the posterior hypothalamus, 5HT turnover was significantly higher in NDI than laying or incubating birds.

The turnover of 5HT and DA measured by amine accumulation after pargyline treatment closely agrees with that assessed by measuring amine:metabolite ratios.

4.4 Conclusion : The Influence of Monoamines in the Control of Prolactin and Luteinizing Hormone Secretion

In the present series of experiments, situations in which LH secretion was high and prolactin secretion low (i.e. in laying and NDI birds), were associated with lowered turnover of dopamine, 5-hydroxytryptamine and adrenaline in the anterior hypothalamus and a low turnover of adrenaline in the posterior hypothalamus relative to those in incubating birds. Only in NDI birds was the high level of LH secretion and low level of prolactin secretion associated with no detectable turnover of noradrenaline and adrenaline in the anterior hypothalamus and adrenaline in the posterior hypothalamus. In incubating birds, where the secretion of LH was low and the secretion of prolactin high, the turnover of adrenaline, dopamine and 5-hydroxytryptamine in the anterior hypothalamus and the turnover of adrenaline in the posterior hypothalamus were high.

The inverse relationship between plasma levels of prolactin and LH during nest deprivation (Figure 8) and during the transition between laying and incubating (Lea et al. 1981), suggests a link between the mechanisms controlling prolactin and LH secretion. The time taken for these hormonal changes to occur suggests the operation of a long-term

process governing the transition between laying and incubation, and a short-term process responsible for the acute changes seen during nest deprivation.

Previous studies, mentioned earlier (section 1.2.2), suggest that 5HT stimulates prolactin secretion and inhibits LH secretion, and indicate that dopamine inhibits, while noradrenaline and adrenaline stimulate the secretion of both prolactin and LH. However these suggestions are based on the effects of pharamacological responses which may not reflect the actions of physiological mechanisms. From the present observations, it would seem that no single monoamine neurotransmitter is solely responsible for the regulation of prolactin or LH secretion. Instead the endocrine changes must be ascribed to net effect of changes in functional activity of several monoaminergic neurotransmitters. Changes in the activity of 5-hydroxytryptamine and noradrenaline in the posterior hypothalamus and noradrenaline in the anterior hypothalamus may be associated with rapid changes in prolactin and LH secretion, while changes in activity of adrenaline, dopamine and 5-hydroxytryptamine in the posterior hypothalamus may be associated with the longer term endocrine changes associated with broodiness.

It is fully acknowledged that an unequivocal assessment of the role of monoamines in the control of LH and prolactin secretion in terms of differential turnover in different endocrine states is difficult. Thus, it can not be assumed that the correlation between monoamine turnover index and plasma hormone levels implies causality, because of the likely participation of hypothalamic monoaminergic systems in the regulation of aspects of hypothalamic function other than the control of prolactin and LH secretion. It is also acknowledged that monoamine turnover rate reflects the activity of the pre-synaptic neurone and that the relationship between released transmitter and its post-synaptic action may also change in different endocrine states. It was for this reason that differences in neurotransmitter-receptor characteristics were investigated in the hypothalami of laying and broody hens. Since the function of hypothalamic, 5HT and dopamine neurones seem to be most

affected by the transition from laying to broodiness the hypothalamic neurotransmitter-receptor systems for 5HT and dopamine were chosen for further study.

5.0: 5HT AND DOPAMINE RECEPTORS IN THE HYPOTHALAMUS AND PITUITARY GLAND

In the previous section the functional activity of aminergic neurones in the bantam hypothalamus was assessed in terms of neurotransmitter turnover. Another equally important component of overall functional activity is the ability of the post-synaptic cell to respond to these neurotransmitters after their release from nerve terminals. The physical link between the neurotransmitter and the mechanisms which cause electrical and biochemical changes within the target cell is a specific receptor. Therefore the responsiveness of the target cell to a neurotransmitter is initally dependent on its complement of receptors for that transmitter. Receptors are regulated by a variety of factors including the rate of neuronal firing and neurotransmitter release, hormones from the ovary and pituitary gland, and by other environmental and neurotrophic influences (see section 1.5).

The correlation between changes in 5-hydroxytryptamine and dopamine turnover in the bantam hypothalamus and the plasma levels of LH and prolactin in broody and laying birds prompted a study of putative 5-hydroxytryptamine and dopamine receptors in the hypothalamus and pituitary gland. Putative receptors were identified by study of the kinetics of radioligand binding in vitro. It is generally accepted (Richelson, 1984) that binding sites can be equated with receptor sites only when the binding can be shown to be associated with a physiological or pharmacological event, but seldom has this criterion been met. In much of the literature, 'binding site' and 'receptor' are used synonymously. This apparent anomaly arises because the primary biochemical effect of receptor activation has been established for only a few receptor types in homogeneous and readily available neural tissues (eg. calf cortex: Burt et al. 1976; and cultured neuroblastoma cells: Richelson, 1979). The likely heterogeneity of receptor types in the hypothalamus and the lack of knowledge of molecular events following receptor activation in this tissue, makes correlation of radioligand binding with a physiological event difficult. The use of the correlation of pharmacological manipulation of behavioural or endocrine events and radioligand binding as a means of receptor identification, suffers from the likelihood that such events are the manifestation of interacting systems of neurones. These difficulties

become further pronounced when interpreting radioligand binding to the avian brain, because what is known of receptor physiology and the effects of receptor activation in the mammalian brain may not be applicable.

In the light of these uncertainties the data presented for radioligand binding to the hypothalamus and pituitary gland of the the bantam hen were interpreted with caution.

5.1 5-hydroxytryptamine (5HT) Binding Sites in Bantam Brain

Direct study of 5-HT receptors in the central nervous system began in the late 1960's (Marchbanks, 1966). The first successful demonstration of binding to the putative 5-HT receptor in homogenates of brain was performed by Bennett and Aghajanian (1974) using ^{3}H-lysergic acid diethylamide. Since then the availability of tritiated 5HT receptor ligands has prompted many studies of the kinetics and distribution of the 5HT receptor(e.g.; ^{3}H-5-hydroxytryptamine: Bennett and Snyder, 1975 and 1976; Fillion et al. 1978; Nelson et al. 1978; ^{3}H-lysergic acid diethylamide: Bennett and Snyder, 1975; Lovell and Freidman, 1976; Leyson and Laduron, 1977; ^{3}H-methiothepin; Nelson et al. 1979; ^{3}H-PAT; Gozlan et al. 1983). Subtle differences in the binding characteristics of ^{3}H-5HT, ^{3}H-spiperone and ^{3}H-LSD led to the proposition that there are two sub-types of 5HT receptor in the mammalian brain (Peroutka and Snyder, 1979; Seeman et al. 1980). The $5HT_1$ (agonist) receptor preferentially binds ^{3}H-5HT and the $5HT_2$ (antagonist) receptor preferentially binds ^{3}H-spiperone. Tritiated - LSD was chosen as the ligand in the following experiments because of its ability to bind to both $5HT_1$ and $5HT_2$ receptors with equal affinity in rat brain (Peroutka and Snyder,1979). The physiological significance of these two types of 5HT receptor is currently being investigated (Peroutka and Snyder, 1983).

Specific ^{3}H-LSD binding, assessed by incubation in the presence or absence of 1 µM d-LSD, to homogenates of bantam hypothalami was found to be temperature dependent (Fig. 14A) and reached equilibrium within

30 minutes (Fig. 14B). These preliminary experiments allowed the selection of optimum incubation conditions (39°C for 30 minutes), which were used throughout later experiments. The amount of specific binding to crude synaptic membrane fractions (CSM) prepared from homogenates of hypothalami, suggested that the specific binding sites were mostly localised on cellular membrane fragments rather than in the nuclear (P1) or microsomal (RP2) cell components (Fig. 14C). As only a low proportion of specific binding occurred in P1 and RP2 fractions the total particulate pellet was used in all further studies. Specific (^{3}H)-LSD binding was found to be linear over the range of concentrations of total particulate preparation routinely used, ($p<0.01$, F test after linear regression, Fig. 14D).

The parameters of ^{3}H-LSD binding to hypothalamic homogenates are shown in Fig. 15. Specific binding was found to be saturable within the low nanomolar range and was monophasic(Fig. 15A). Non-saturable binding was linear within the range tested. Competitive inhibition studies (Fig. 15B) demonstrated that the radioligand was bound to 5HT binding sites. Specific ^{3}H-LSD binding was displaced by low concentrations of 5HT, d-LSD and the 5HT agonist quipazine, in a parallel fashion. Dopamine only began to inhibit specific ^{3}H-LSD binding at high concentrations and displacement did not parallel the other ligands used. The data presented here established the existence of specific 5HT binding sites in the bantam hypothalamus which could be reliably measured in the assay system.

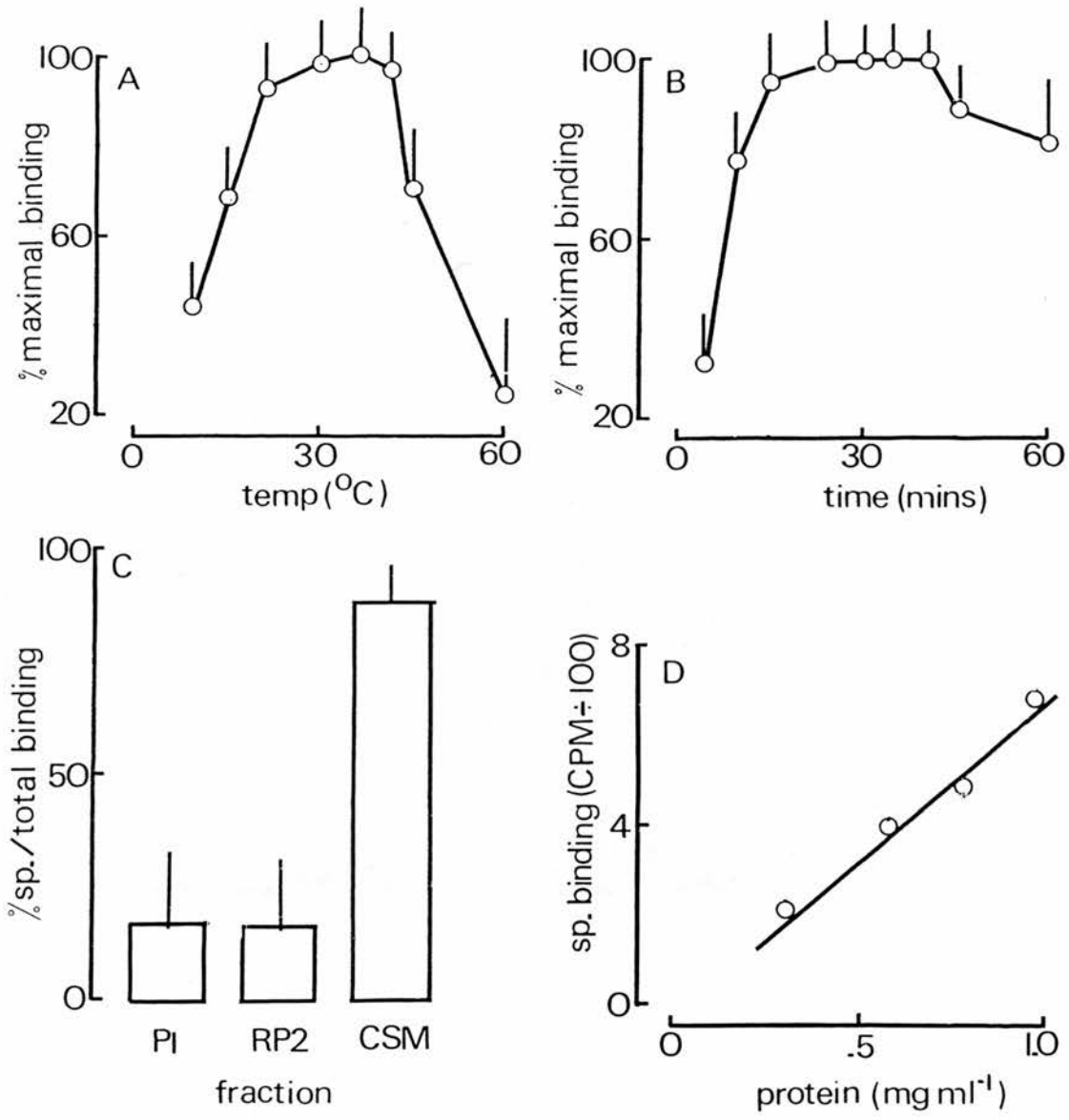


Figure 14: ^{3}H-LSD binding to homogenates of bantam hypothalami. A:The effect of temperature on the specific binding of 5mM ^{3}H-LSD in a 30 minute incubation (mean + S.E.M., n=6). B:The effect of incubation time on specific ^{3}H-LSD binding at 39° C (mean + S.E.M., n=8). C:Specific ^{3}H-LSD binding to the crude nuclear fraction (P1), the residual mitochondrial fraction (RP2) and crude synaptic membrane fraction (CSM) in incubations of 30 mins at 39° C (means + S.E.M., n=8). D:Specific ^{3}H-LSD binding to various concentrations of homogenate in incubations of 30 minutes at 39° C. Linear regression gave the line of best fit ($p \leq 0.01$, F-test, df=29)

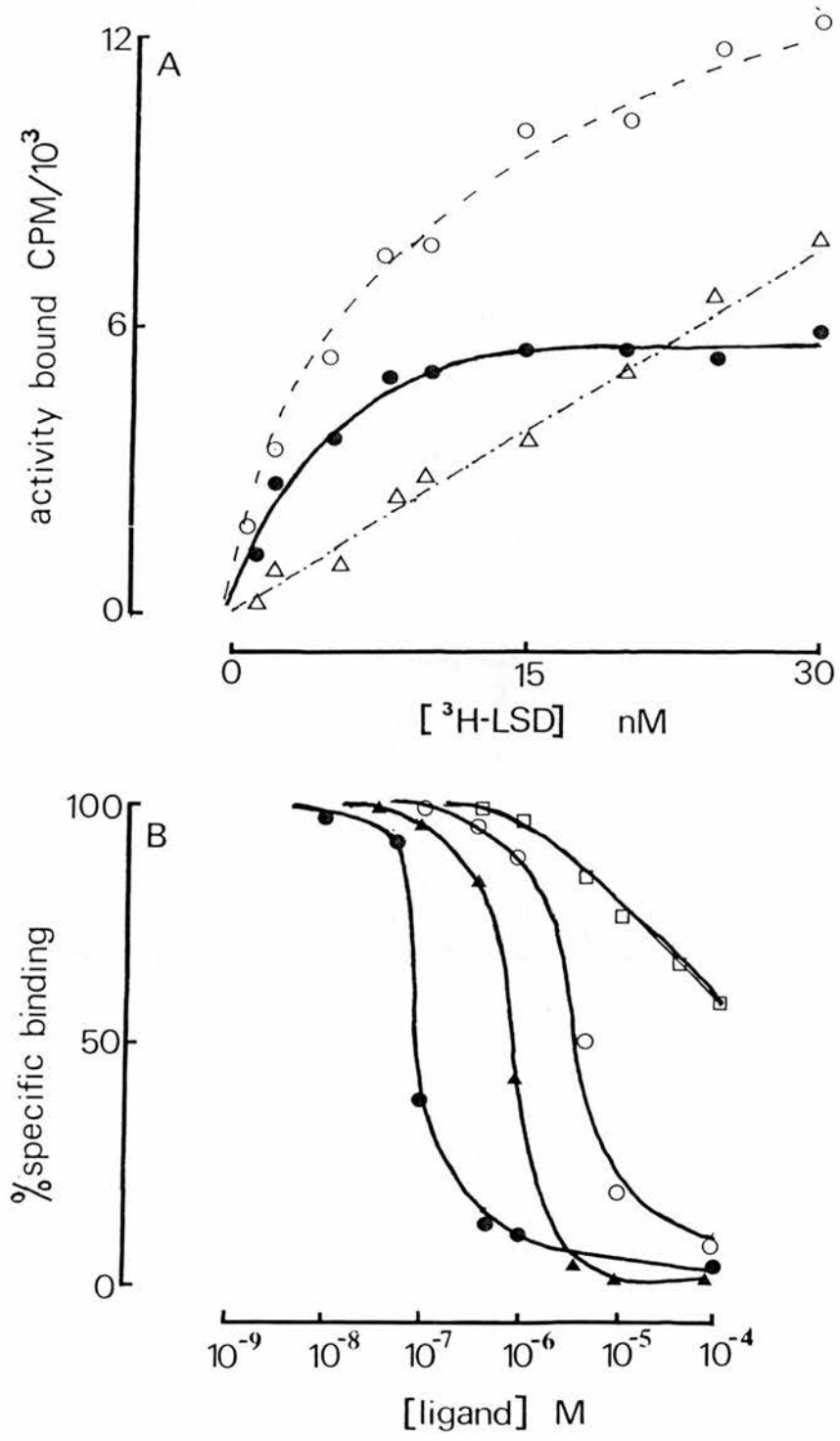


Figure 15: ^{3}H-LSD binding to homogenates of bantam hypothalami. A:Result of a typical saturation study, showing plots of calculated specific binding (●), and actual non-specific (△) and total binding (○). B: Result of a typical inhibition study, showing the effect of various concentrations of 5HT (●), d-LSD (▲), quipazine (○) and dopamine (□) ( ). In both cases the points are the mean of 4-6 determinations. The S.E.M's were less than 5% (not shown).

5.2 Dopamine Binding Sites in the Bantam Brain

An intensive study of dopamine receptors in the mammalian brain and pituitary gland was stimulated by several discoveries. These include the discovery that hyperprolactinaemia can be treated by the administration of dopamine agonists and that the tuberoinfundibular dopamine neuronal system plays a key role in the inhibitory control of prolactin secretion. Also, it was found that Parkinson's disease could be treated with L-DOPA and that it occurs after pathological destruction of nigro-striatal dopaminergic pathways. On the basis of this research, four subtypes of dopamine receptor have been identified (Seeman, 1982; Cronin, 1982; Creese et al. 1983). However only the 'D_2' site fulfils all the criteria for a receptor, and is associated with, or mediates the majority of dopaminergic responses in mammals (Seeman, 1982).

Studies on the binding of ^{3}H-domperidone to mammalian nervous tissue have established that it is selectively bound by dopamine receptors (Baudry et al . 1979; Sokoloff et al. 1980; Watling and Iversen, 1981). Because of its low lipophilicity, ^{3}H-domperidone has the advantage over other dopaminergic ligands of having lower levels of non-specific binding.Consequently it was used throughout the following experiments.

Initial investigations demonstrated that specific binding of ^{3}H-domperidone binding to bantam hypothalami, assessed by incubation in the presence and absence of 1μM domperidone, was temperature dependent (Fig 16A) and reached equilibrium after 20 minutes (Fig 16B). The bulk of specific binding was contained in the crude synaptic membrane (CSM) preparation with low, often undetectable, specific binding to nuclear (P1) and mitochondrial (RP2) fractions (Fig. 16C). Specific ^{3}H-domperidone binding to total particulate pellet preparations of hypothalami was also found to be linear over the range of concentrations routinely used ($p<0.01$, F test after linear regression, Fig. 16D).

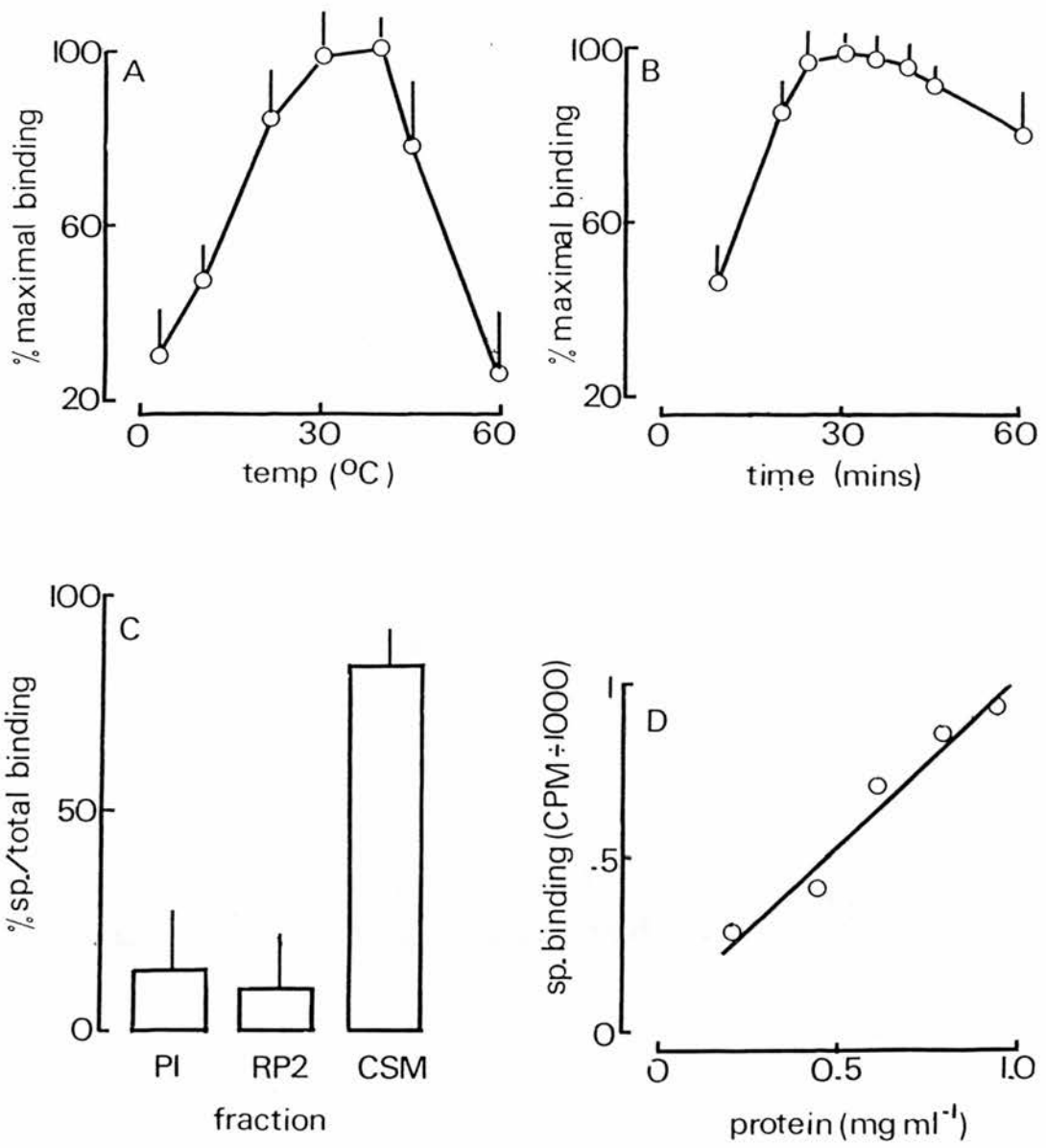


Figure 16: ^{3}H-domperidone binding to homogenates of bantam hypothalami. A: The effect of temperature on the specific binding of 3mM ^{3}H-domperidone in a 30 minute incubation (means ± S.E.M., n=6). B:The effect of incubation time on specific ^{3}H-domperidone binding at 39° C (means ± S.E.M., n=6). C:Specific ^{3}H-domperidone binding to the crude nuclear fraction (P1), the residual mitochondrial fraction (RP2) and crude synaptic membrane fraction (CSM) in incubations of 30 minutes at 39° C (means ± S.E.M., n=8). D:Specific ^{3}H-domperidone binding to various concentrations of homogenate in incubations of 30 minutes at 39° C. Linear regression gave the line of best fit ($p<0.01$, f-test, df=29)

Figure 17A shows that specific ^{3}H-domperidone binding to homogenates of hypothalami was saturable at low nanomolar concentrations of ligand and was monophasic. It was evident from the abilities of various ligands to inhibit specific binding that ^{3}H-domperidone labels dopamine binding sites (Fig 17B). Dopamine, domperidone and (+) butaclamol all display parallel displacement of ^{3}H-domperidone in low concentrations. The large difference in potency (approximately 1000 fold) between (+) butaclamol and (-) butaclamol, demonstrates the stereospecificity of the ^{3}H-domperidone binding site. Only marginal displacement of ^{3}H-domperidone binding occurred in the presence of high concentrations of 5-HT.

The data presented here established the existence of specific dopamine binding sites in the bantam hypothalamus and showed that they were measurable in the assay system.

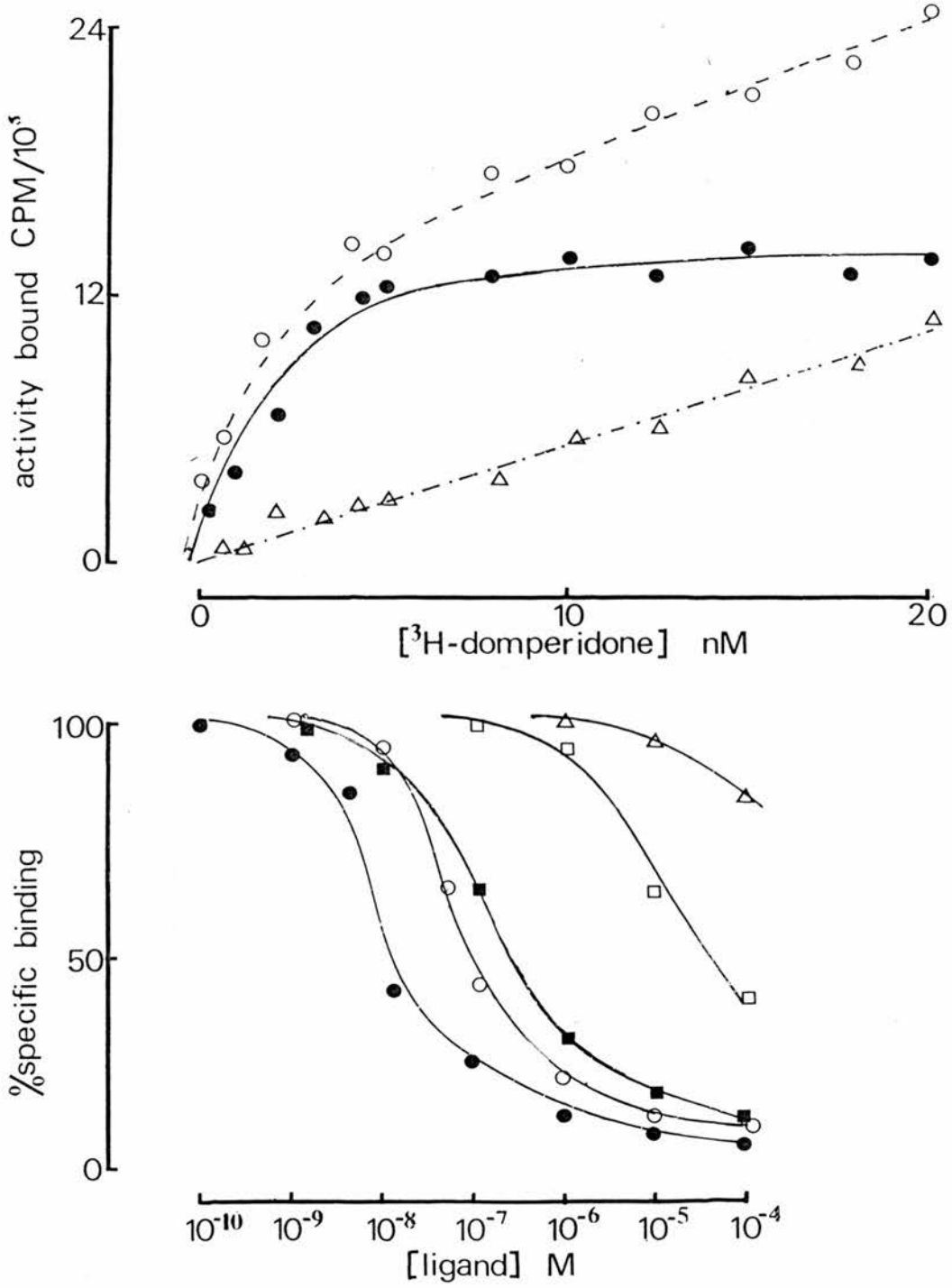


Figure 17: ^{3}H-domperidone binding to homogenates of bantam hypothalami. A: Result of a typical saturation study, showing plots of calculted specific binding (●), and actual non-specific (△) and total binding (○). B: Result of a typical inhibition study, showing the effect of various concentrations of dopamine (●), domperidone (○), +butaclamol (■), -butaclamol (□), and 5HT (△). In both cases the points are the mean of 4-6 determinations. The S.E.M's were less than 5% (not shown).

5.3 Receptors in the Anterior Pituitary Gland

Studies of prolactin release in mammals both *in vivo* and *in vitro* suggest that dopamine is a prolactin release-inhibiting factor (see section 1.2). The use of radioligand binding techniques has proved the existence of dopamine receptors in the mammalian anterior pituitary gland and that their activation is inversely correlated with prolactin secretion (review: Cronin, 1982). There is also evidence for an inhibitory effect of dopamine at the level of the pituitary gland in the control of prolactin secretion in birds. Thus, studies on isolated chicken pituitary glands *in vitro* show that the stimulation of prolactin release from the isolated chicken pituitary gland is inhibited by dopamine (Harvey et al. 1982). Further, 5HT has been shown to be a potent prolactin releasing agent in birds *in vivo* (Rabii et al. 1981), with a direct effect on the isolated pituitary gland (Border and Chadwick, 1977). If these effects are physiologically relevant to the control of prolactin secretion in the bantam hen, then the anterior pituitary gland should contain specific receptors for 5HT and dopamine.

5.3.1 Lack of Demonstrable Specific 5HT Binding Sites in the Bantam Anterior Pituitary Gland

In several studies, initially using ^{3}H-5HT and subsequently ^{3}H-LSD, no specific binding could be detected in preparations of anterior pituitary glands from either laying or incubating bantams. Large amounts of non-saturable binding was observed which was not inhibited by 5HT or d-LSD even in high concentrations (10^{-2}M). The complete lack of any specific 5HT binding sites in the bantam anterior pituitary gland suggests that the prolactin releasing effect of exogenous 5HT on the pituitary gland *in vitro* has little or no relevance to the physiological control of prolactin secretion. The direct stimulatory action of 5HT on prolactin secretion from the chicken pituitary gland *in vitro* may be due to a non-specific membrane effect of the transmitter.

5.3.2 Dopamine Binding Sites in the Anterior Pituitary Glands of Laying and Incubating Bantam Hens

The ability of homogenates of anterior pituitary glands from laying and incubating bantam hens to bind ^{3}H-domperidone was assessed. Saturable specific binding occurred in both tissues and inhibition studies suggested that ^{3}H-domperidone was bound to specific DA binding sites(Figs. 18,19). The binding curves(A) showed a monophasic saturation of specific binding sites at low nanomolar concentrations of ligand. From the Scatchard plots of these data (B) it was possible to define the apparent dissociation constant of the binding (K_D) and also the maximal number of binding sites in the tissues (Bmax). The Hill plots (C), derived from Scatchard analysis, also yield an estimate of the apparent dissociation (K_D') and of the Hill coefficient (H_n), which assesses the homogeneity of binding. Dopaminergic ligands were found to displace specific ^{3}H-domperidone binding in a parallel fashion (C). No differences were observed between pituitary preparations from laying or incubating hens in the characteristics of the displacement curves for several ligands used to investigate the specificity of the binding. The kinetic parameters of these DA binding sites from repeated independent experiments are given in table 2. The binding sites in the pituitary glands of laying and incubating birds had similar affinities (K_D and K_D' values not significantly different) and were homogeneous in that the Hill coefficients (Hn) did not significantly differ from unity.

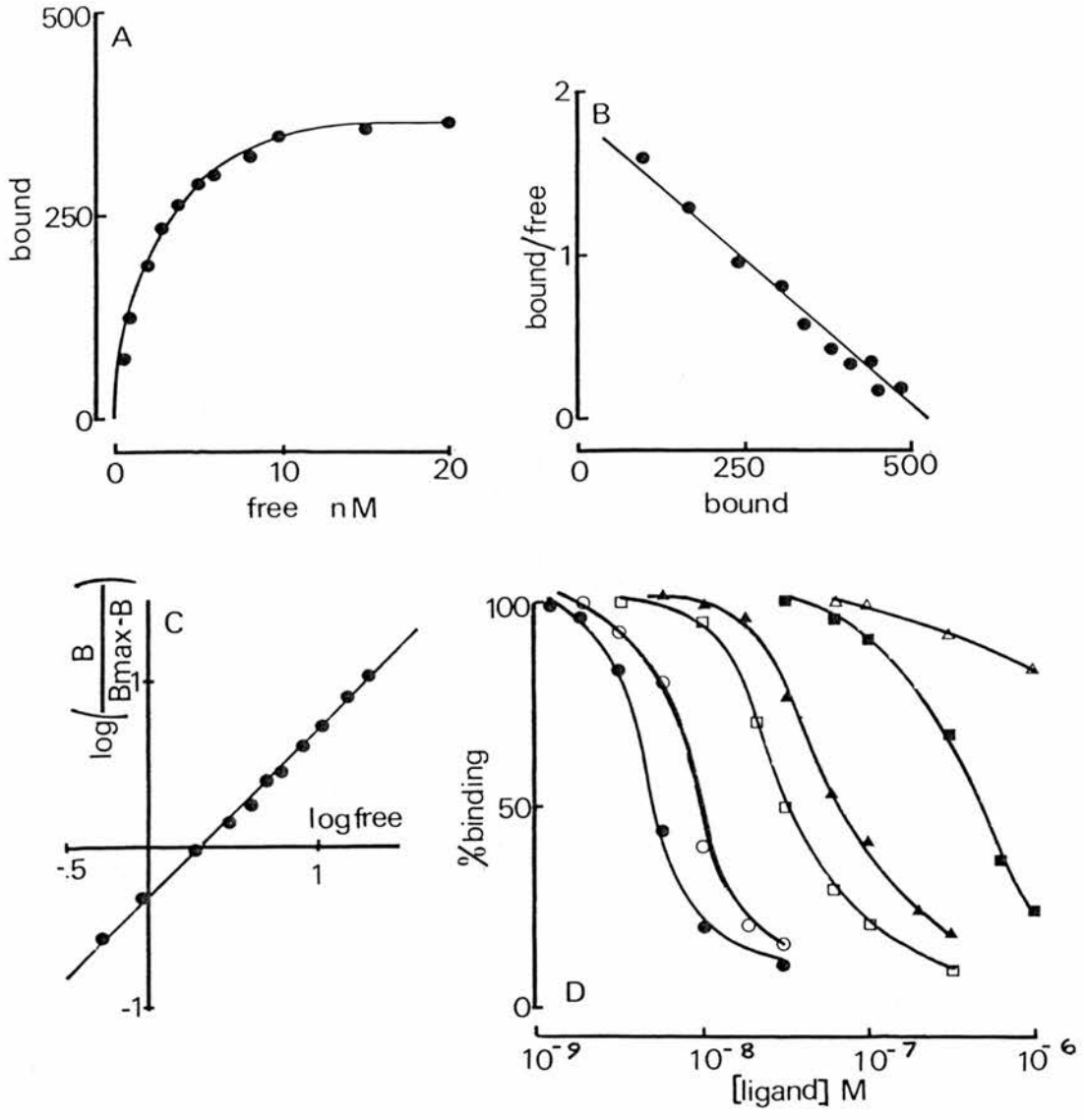


Figure 18: Dopamine binding sites in homogenates of anterior pituitary glands of laying bantam hens. A:An example of the typical saturation profile of specific binding. B:Scatchard analysis of saturation data. C: Hill plot of saturation data using the Bmax derived from Scatchard analysis. D:Inhibition of specific binding by dopamine (●), domperidone (○), apomorphine (□), + butaclamol (▲), - butaclamol (■), and 5HT (△) (means of 6 determinations S.E.M. less than 5%).

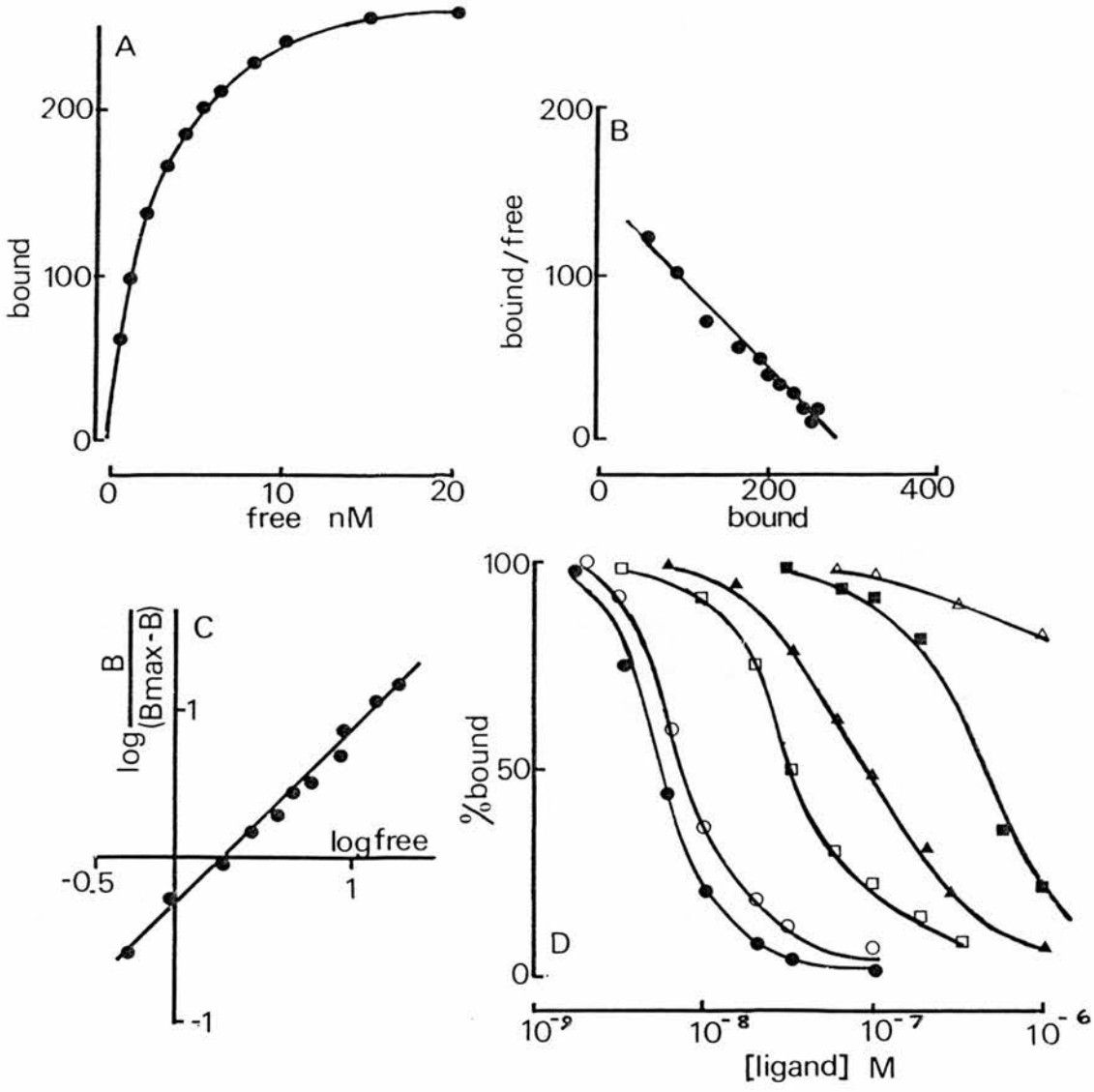


Figure 19: Dopamine binding sites in homogenates of anterior pituitary glands of incubating bantam hens. A:An example of the typical saturation profile of specific binding. B:Scatchard analysis of saturation data. C: Hill plot of saturation data using the Bmax derived from Scatchard analysis. D:Inhibition of specific binding by dopamine (●), domperidone (○), apomorphine (□), + butaclamol (▲), - butaclamol (■), and 5HT (△) (means of 6 determinations S.E.M. less than 5%).

Table 2: Dopamine binding sites in the anterior pituitary gland.
The following table was compiled from the results of repeated (n=3-5) independent saturation and inhibition studies. The apparent dissociation constant (KD) and number of binding sites (Bmax) were calculated by Scatchard analysis. Another estimate of KD (KD^{1}) and the Hill number were also calculated. The concentration at which an unlabelled ligand displaced 50% of the specific binding (IC50) was calculated for dopamine (DA), domperidone (DOM), +butaclamol (+BUT), -butaclamol (-BUT), apomorphine (APO), and 5-hydroxytryptamine (5HT). Where appropriate results are expressed as the mean ±SEM (**-$p<0.001$, unpaired t-test)

	KD (nM)	Bmax (fmolmg-1)	KD^{1} (nM)	Hc
Laying	2.19±0.17	420±32	2.17±0.2	0.92±0.10
Incubating	1.87±0.21	238±46**	1.89±0.3	0.97±0.09

	IC50 (nM)					
	DA	**DOM**	**+BUT**	**APO**	**-BUT**	**5HT**
Laying	7	9	100	700	47000	100000
Incubating	8	9	100	850	52000	100000

The potencies of ligands which inhibit binding were the same in pituitary gland preparations from laying and incubating birds, and the low potency of (-)butaclamol suggests that the binding sites were stereospecific. The only detectable difference between preparations of anterior pituitaries from laying and incubating birds was the presence of significantly higher numbers of binding sites (Bmax) in the laying gland($p<0.01$, unpaired t-test).

An interesting analogy exists between mammalian prolactin secreting pituitary tumours and the incubating chicken pituitary gland, in that both secrete large quantities of prolactin. The tumours are ,however,refractory to the inhibitory effect of dopamine, suggesting a change in the mechanism of dopamine action (Cronin et al. 1981). Radioligand binding studies have shown that the tumours contain dopamine receptors which have a similar affinity to those found in normal glands (MacLeod and Cronin 1983). The pathological nature of such tumours does not allow the comparison of the absolute number of binding sites they contain, with that in the normal gland. From the data presented here, it would seem that dopamine binding sites in the bantam anterior pituitary must be 'down-regulated' in situations of chronic prolactin release to account adequately for their decrease in numbers at a time when prolactin release is high. This may reflect a change in the morphology of the pituitary prolactin cells or may be the result of an adaptive process minimising the possible prolactin release-inhibiting effect of dopamine in the incubating bird.A similar 'down-regulation' of dopamine receptors may account for the apparent refractoriness of the mammalian pituitary tumour.

5.4 Receptors in the Hypothalamus

The correlation between increased turnover of hypothalamic 5HT (Section 4.3) and the development of broodiness in the bantam hen, together with the evident absence of 5HT binding sites in the pituitary gland (Section 5.2.) suggests a hypothalamic locus for the action of 5HT. An ~~possible~~ influence of dopamine through the hypothalamus is also

possible. The characteristics of dopamine and 5HT binding sites in the hypothalamus were studied in birds at different stages of the reproductive cycle.

5.4.1 Hypothalamic 5HT Binding Sites During the Reproductive Cycle

The binding of ^{3}H-LSD to preparations of anterior and posterior hypothalami was compared in non-laying, laying, and incubating bantam hens. Specific, saturable ^{3}H-LSD binding, with displacement characteristics suggesting it was bound by specific 5HT binding sites, was found in both parts of the hypothalamus taken from birds at different stages of the reproductive cycle. Scatchard plots of the binding curves for all tissues except anterior hypothalami from laying hens, were linear, suggesting the existence of a single type of 5HT binding site. In contrast, the Scatchard plots of the binding curve for preparations of anterior hypothalamus from laying hens were non-linear. These Scatchard plots were readily resolved into two components suggesting the existence of two binding sites (Keightley et al. 1983). A Hill plot of the data confirmed the difference in apparent affinity. Typical Scatchard plots of data from saturation curves from experiments using anterior hypothalami of laying and incubating bantam hens are presented in figure 20A and 20B respectively.

The inhibition of specific ^{3}H-LSD binding to preparations of anterior hypothalami from laying birds by 5HT was bimodal, whereas the inhibition of binding to preparations of posterior hypothalami from laying birds and to anterior and posterior hypothalami from incubating birds was monophasic. This was further evidence of the existence of two 5HT binding sites of different affinities in the anterior hypothalamus of the laying bird. Examples of data from inhibition experiments using anterior hypothalami from laying and incubating hens are shown in figure 20 (C & D).

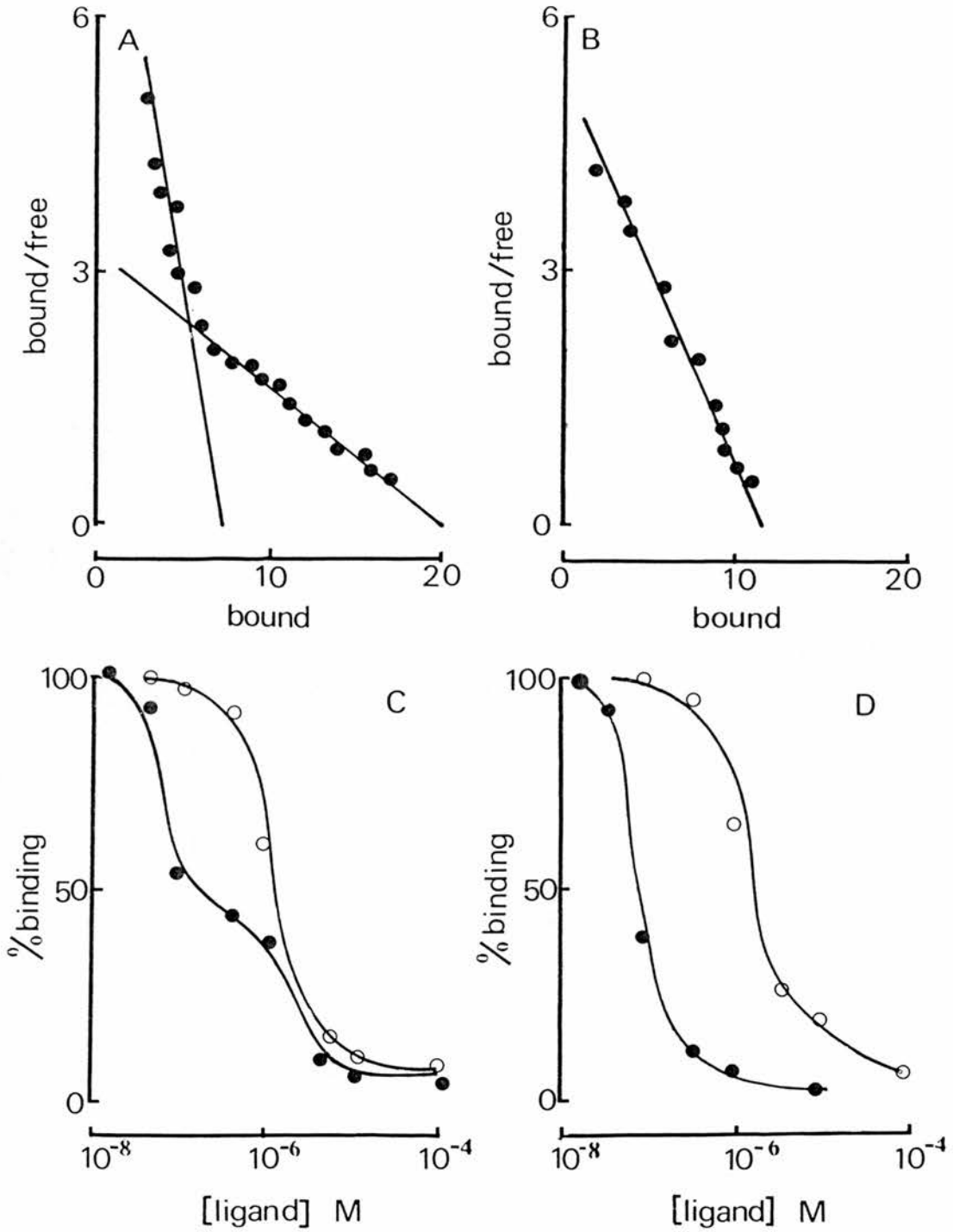


Figure 20: 5HT binding sites in the anterior hypothalamus. A:Scatchard plot of saturation data for specific ^{3}H-LSD binding to homogenates of anterior hypothami from laying bantam hens. B:Scatchard plot of saturation data for specific ^{3}H-LSD binding to homogenates of anterior hypothalami from incubating bantam hens. C:Inhibition of specific binding to homogenates of anterior hypothalmi from laying birds by 5HT (●) and d-LSD (o) (means of 12 observations, S.E.M.< 5%). D:Inhibition of specific binding to homogenates of anterior hypothalami from incubating birds by 5HT (●) ad - LSD (o) (means of 12 observations, S.E.M.'s<5%)

In the absence of additional information concerning the possible differential ligand preferences of these two co-existing 5HT binding sites in the anterior hypothalamus of the laying bird, their interrelationship can be expressed in a number of ways :

1. The binding sites may represent two independent receptor types, and can be described as follows;

$$R_1 + L = R_1L$$
$$R_2 + L = R_2 L$$

Where R_1 and R_2 are receptors and L is the ligand.

2. The binding sites may be part of the same receptor and their interaction may show some degree of cooperativity. In its simplest form this interaction can be described as follows;

$$R_2 + L = R_2L + L = R_2L_2$$

Apart from sequentially cooperative interaction, like that described above, more complex interactions involving concerted cooperativity and further receptor activation after initial binding may also occur (Boeynaems and Dumont, 1980).

An attempt was made to determine whether the two 5HT binding sites were independent, by investigating the possibility that one of the binding sites was located on neurones containing 5HT. This was achieved by treating laying bantam hens with the 5HT neurotoxin p-chloramphetamine(pCA).

Chronic treatment with p-chloramphetamine (pCA) resulted in a 72% decrease in 5HT, and the complete disappearence of 5-hydroxyindole acetic acid in the hypothalamus (Fig. 21A). The catecholamine content of the hypothalamus was not affected. These findings are consistent with the assumption that pCA acted as a specific 5HT neurotoxin and

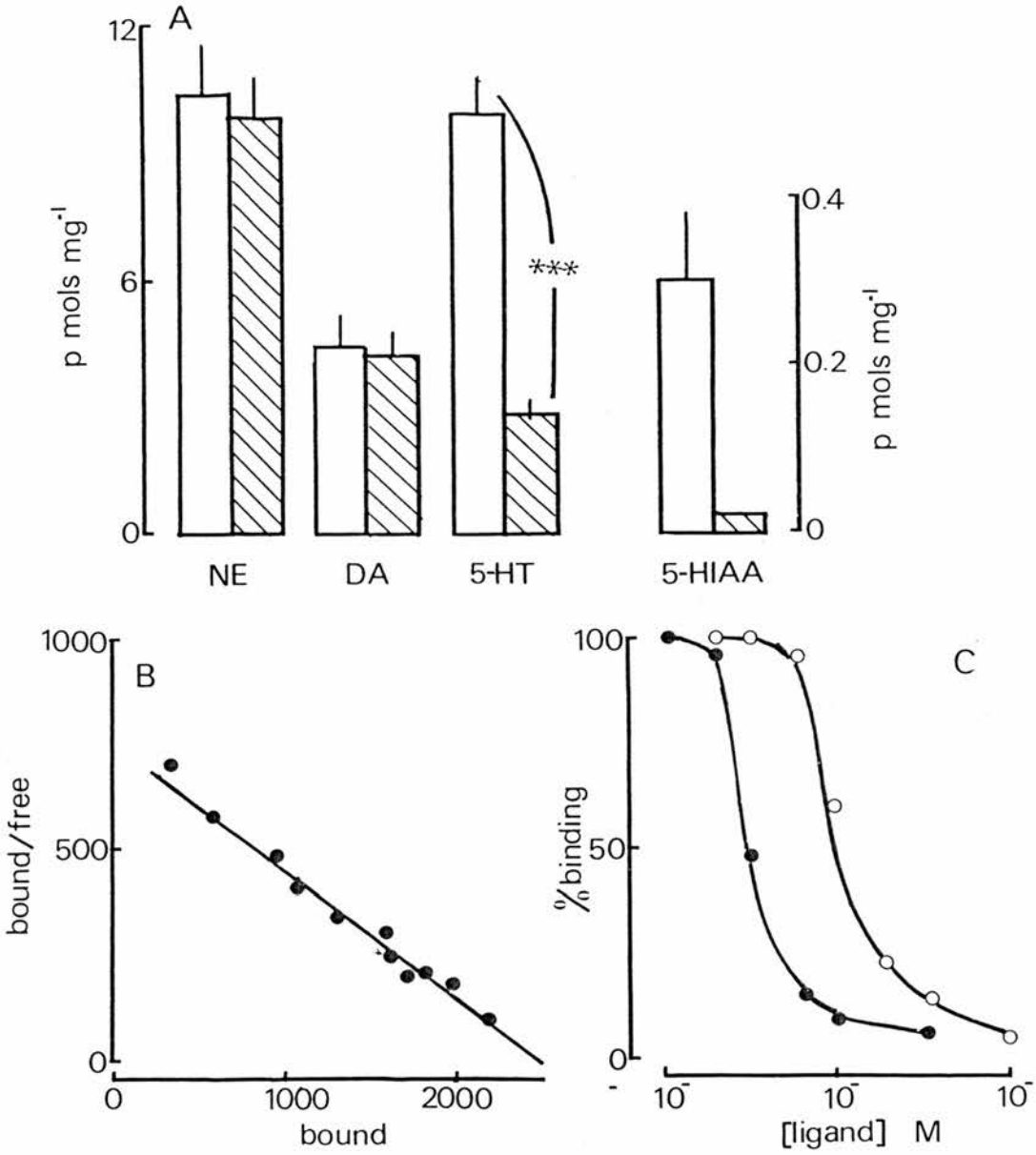


Figure 21: A:concentrations of noradrenaline (NE), dopamine (DA), 5-hydroxytryptamine (5HT) and 5-hydroxyindole acetic acid (5H1AA) in anterior hypothalami from laying bantam hens treated with saline (□) or p-chloramphetamine (▨). Results are the means + SEM of 6 observations (***-p<0.001, unpaired t-test). B:scatchard plot of saturation studies of ^{3}H-LSD binding to homogenates of anterior hypothalami from PCA treated laying hens. C:inhibition, by 5HT (●) and d-LSD (○), of specific ^{3}H-LSD binding to homogenates of anterior hypothalami from PCA treated laying hens (means of 8 observations, SEM less than 5%).

destroyed hypothalamic 5HT neurones. Repeated studies of ^{3}H-LSD binding to preparations of anterior hypothalami from pCA treated birds consistently showed saturable specific binding which gave a linear plot on Scatchard analysis (Fig. 21B). Further evidence that only one 5HT binding sites was present in this tissue comes from the monophasic nature of the inhibiton of specific ^{3}H-LSD binding by 5HT(Fig. 21C). However, the kinetics of the remaining binding sites did not resemble either of those which occurred in anterior hypothalami from laying birds. This may have been due to adaptive changes in the regulation of 5HT receptors in response to decreased 5HT tone. These findings suggest that one of the two 5HT binding sites in anterior hypothalami from laying birds is located on 5HT neurones, while the other is located post-synaptically. Therefore it is likely that the two sites represent independent putative 5HT receptors which conform to simple occupancy theory.

The results of repeated independent studies of ^{3}H-LSD binding to preparations of hypothalami are given in Table 3. In summary, a homogeneous population of 5HT binding sites of similar affinity and number were found in the posterior hypothalamus of laying, incubating and out-of-lay birds. In the anterior hypothalamus, a second population of 5HT binding sites possibly located on 5HT neurones, was observed only in laying birds. Other binding sites for 5HT were present in preparations of anterior hypothalami from laying, incubating and out-of-lay birds, and had similar affinities and numbers.

5.4.2 Hypothalamic Dopamine Binding Sites During the Reproductive Cycle

The specific binding of ^{3}H-domperidone to anterior and posterior hypothalami was compared in preparations from laying and incubating bantam hens. Specific, saturable ^{3}H-domperidone binding with displacement characteristics suggesting dopamine binding sites occurred in both parts of the hypothalamus in both physiological states. In repeated experiments Scatchard plots of saturation data were linear. Hill plots of binding data confirmed that ^{3}H-domperidone was bound by a single non-interactive site. The results of repeated independent

Table 3: 5HT binding sites in the hypothalamus.

The following table was compiled from the results of repeated (n=4-6) independent saturation studies. The apperent equilibriation dissociation constant (KD) and the number of binding sites (Bmax) were calculated by Scatchard analysis. The dissociation constant ($KD^{\backslash}$) and the Hill number (Hc) were also calculated by Hill analysis.

	K_D	Bmax	$K_D^{\backslash}$	Hc	n
ANTERIOR HYPOTHALAMUS					
Out-of-lay	2.4±0.34	1270±200	2.5±0.25	1.01±0.17	4
Laying	1.8±0.50	1110±176	2.2±0.16	0.98±0.09	6
	6.7±1.30	2034±239	6.9±2.10	0.99±0.12	6
Incubating	2.3±0.20	1153± 36	2.1±0.13	1.09±0.14	6
pCA treated	3.2±0.49	2535±300	3.2±0.32	1.02±0.14	4
POSTERIOR HYPOTHALAMUS					
Out-of-lay	2.6±0.25	1180±151	2.7±0.30	0.96±0.20	4
Laying	3.2±0.50	1075±147	3.0±0.45	0.97±0.15	6
Incubating	2.9±0.40	1136± 68	3.1±0.26	0.97±0.13	5

studies are given in Table 4. Inspection of the apparent affinity of binding (K_D and K_D^1) and the number of binding sites (Bmax) showed that dopamine binding sites existed throughout the bantam hypothalamus and that they were kinetically similar in both laying and incubating birds.

5.5 Conclusion: Implications of changes in putative 5HT and DA receptors during the reproductive cycle

High affinity binding sites for 5HT occurred in large numbers throughout the bantam hypothalamus. In the anterior hypothalamus of laying birds two kinetically different 5HT binding sites were present. Only one type of 5HT binding site was found in all other tissues studied and the data from pCA treated birds suggest that one of the sites present in the laying anterior hypothalamus was located on 5HT neurones. No specific 5HT binding sites were detected in the anterior pituitary gland.

The presence of 5HT binding sites on 5HT neurones in the anterior hypothalamus of laying birds and their apparent absence in the anterior hypothalamus of incubating birds suggests a mechanism by which 5HT turnover in this area is regulated. If it is assumed that the population of 5HT receptors unique to laying birds consists of pre-synaptic autoreceptors and is responsible for an inhibition of 5HT neurones, then during the transition between laying and incubation the disappearance of this population could result in an increase in 5HT turnover (see section 4). A possible mechanism for the change in 5HT binding sites in the anterior hypothalamus may be related to the drop in gonadal steroid production seen at the onset of incubation (Sharp et al. 1979) or the initial rise in prolactin secretion acting either directly in the hypothalamus or indirectly by inhibiting ovarian steroidogenesis.

High affinity dopamine binding sites were present in low numbers throughout the bantam hypothalamus and no differences in their kinetics were detected between laying and incubating birds. High affinity dopamine binding sites were present in large numbers in the anterior

Table 4: Dopamine binding sites in the hypothalamus.
The following table was compiled from the results of repeated (n=4) independent saturation studies. The apparent equilibriation dissociation constant (KD) and the number of binding sites (Bmax) were calculated by Scatchard analysis. The dissociation constant ($KD^{\prime}$) and the Hill number (Hc) were also calculated by Hill analysis.

	K_D	Bmax	$K_D^{\prime}$	Hc	n
ANTERIOR HYPOTHALAMUS					
Laying	1.5±0.39	66±10	1.46±0.21	0.98±0.04	4
Incubating	1.6±0.29	60±13	1.53±0.30	1.01±0.10	4
POSTERIOR HYPOTHALAMUS					
Laying	1.3±0.39	66±8	1.24±0.29	1.03±0.10	4
Incubating	1.4±0.21	59±11	1.42±0.23	0.97±0.10	4

pituitary glands of laying hens and were also present, although in significantly lower numbers, in the anterior pituitary glands of incubating hens. This difference may also be attributable to changes in the plasma levels of gonadal steroids at the onset of incubation.

6.0 PHARAMACOLOGICAL MANIPULATION OF PROLACTIN SECRETION

The aim of the following experiments was to examine the influence of 5HT on the control of prolactin secretion in laying and incubating bantam hens. Several drugs were used to manipulate 5HT function in vivo and their assumed modes of action are shown in Figure 22. The effect of the 5HT agonist, quipazine, on prolactin release from the isolated pitiutary and pituitary/hypothalamus co-incubations, was also assessed. Prolactin concentrations in plasma samples and in samples of incubation media were assessed by an homologous chicken prolactin radioimmunoassay (Scanes et al. 1976). The prolactin content of selected samples was also measured in the heterologous prolactin radioimmunoassay (McNeilly et al. 1978). The specificity of the homologous radioimmunoassay was investigated after separation of the protein constituents of samples of incubation media by polyacrylamide gel electrophoresis.

6.1. Drug Treatments in vivo

6.1.1 The Effect of Manipulations of 5HT Systems on Plasma Levels of Prolactin measured in the Homologous Radioimmunoassay

In the laying bantam hen, the 5HT agonist, quipazine, stimulated the release of prolactin in a dose related manner (Fig. 23A), confirming previous observations in immature chickens (Rabii et al. 1981) and turkeys (El Halawani et al. 1983). Pre-treatment of the birds with the 5HT receptor blockers methysergide, cyproheptadine, and SQ 10631, reduced the quipazine stimulated release of prolactin (Fig. 23B), showing that 5HT receptor activation was the basis of the prolactin response to quipazine. Prolactin release was also stimulated in laying bantams by injections of the immediate precursor of 5HT, 5-hydroxytryptophan (Fig. 23C). The stimulatory effect of 5-hydroxytryptophan on prolactin release was reduced by pre-treatment of the birds with the 5HT receptor blocker, cyproheptadine, and enhanced by the specific 5HT reuptake blocker, fluoxetine (Fig. 23D). Fluoxetine alone had no effect on plasma levels of prolactin and did not affect the quipazine-induced release of prolactin. These findings

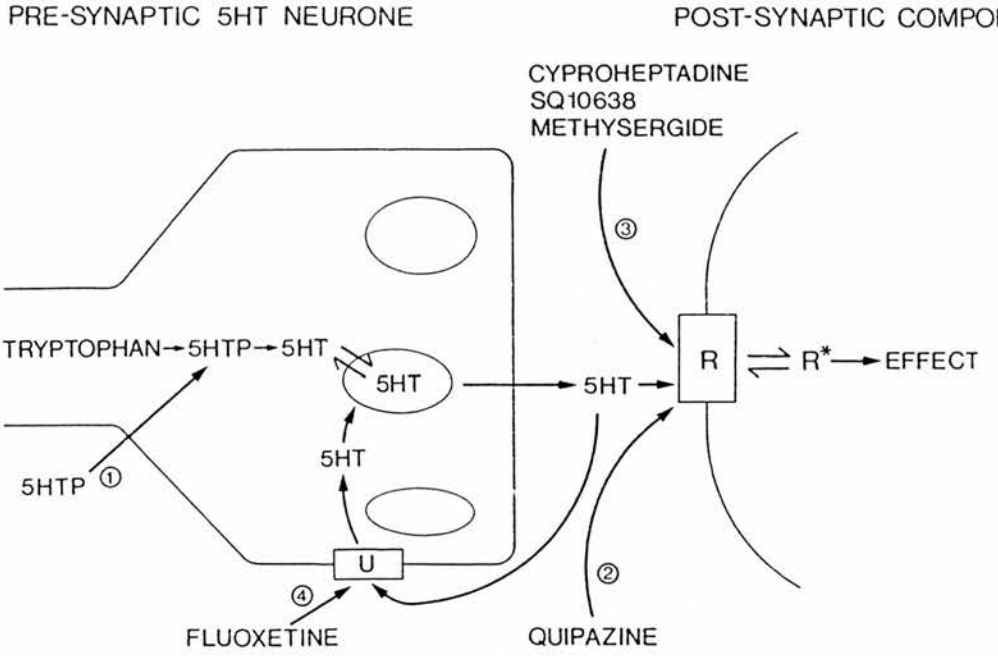


Figure 22: Assumed modes of action of drugs used in vivo
1. Administered 5HTP enhances the release of 5HT by providing additional substrate 5HT synthesis. 2.Quipazine selectively stimulates 5HT receptors thus mimicking the effect of endogenous 5HT. 3. Cyproheptadine, SQ 10638, and methysergide selectively block 5HT receptors thus diminishing the effect of endogenous 5HT. 4.Fluoxetine selectively blocks the specific 5HT reuptake system thus prolonging the synaptic action of endogenous 5HT.

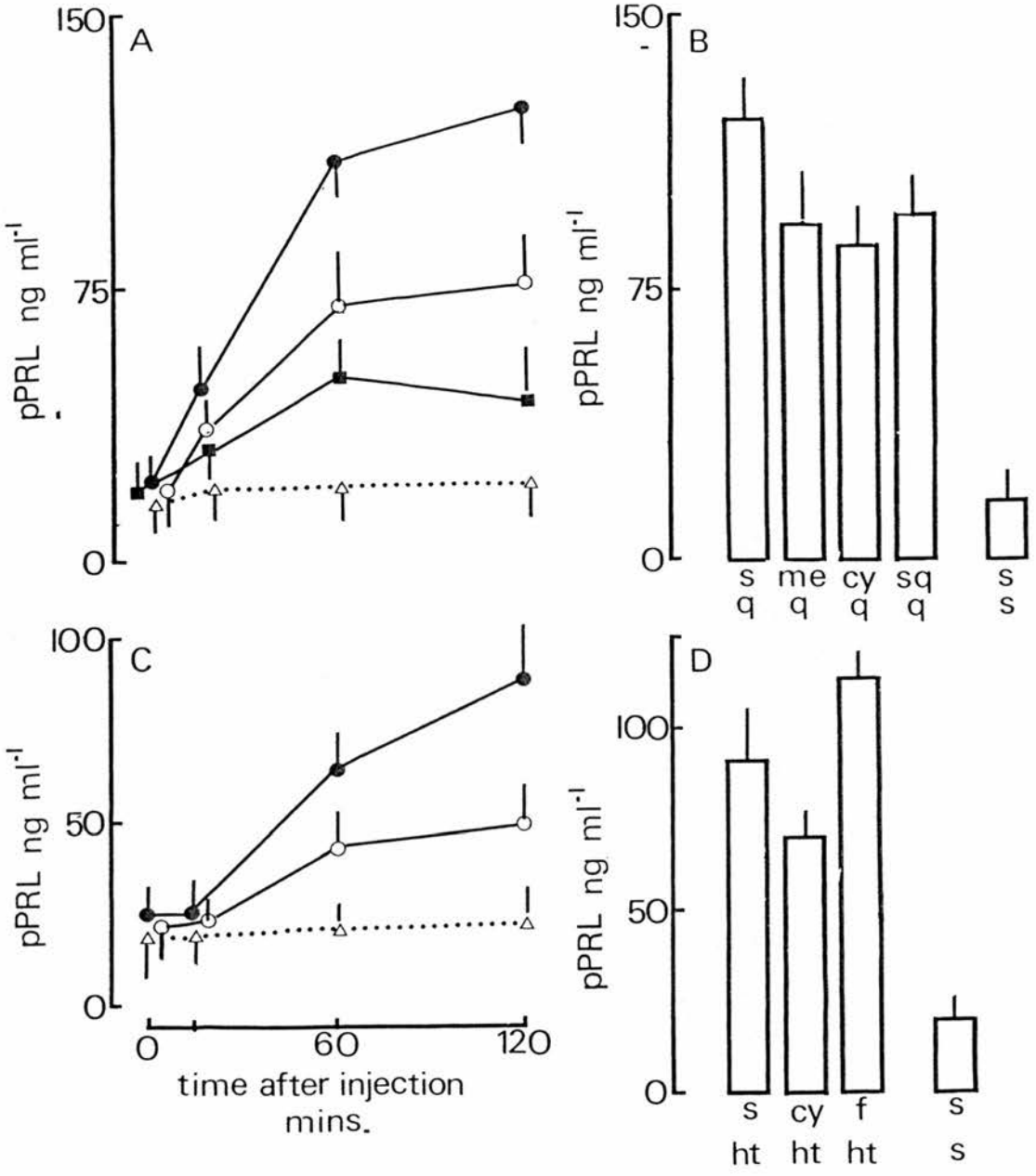


Figure 23: Pharmacological manipulations of plasma levels of prolactin. A: The effect of single intraperitoneal injections of quipazine at 15(●) 10(○),and 5 mg kg^{-1}(■), or saline (△) on plasma levels of prolactin in groups (n=6) laying bantam hens. B: The effect of pre-treatment of laying bantam hens with saline methysergide (10 mg kg^{-1}), cyproheptadime (10 mg kg^{-1}) or SQ 10638 (10 mg kg^{-1}) 30 minutes before an injection of saline or quipazine (15 mg kg^{-1}). Blood samples were taken by venepuncture 2 hours after the second injection. C:the effect of a single intraperitoneal injection of 5-hydroxytryptophan at 50 (●) or 25 mg kg^{-1} (○) or acidified saline (△) on plasma levels of prolactin in groups (n=7) of laying bantam hens. D: The effect of pre-treatment of groups of laying hens (n=6) with saline, cyproheptadine (10 mg kg^{-1}) or fluoxetine (10mgkg^{-1}). Thirty minutes before an injection of acidified saline or 5-HTP (50mgkg^{-1}). Blood samples were taken by venepuncture 2 hours after the second injection.

suggested that 5-hydroxytryptophan acted via 5HT neurones to promote the release of 5HT, which in turn stimulated prolactin secretion. However, a direct effect of 5-hydroxytryptophan on the 5HT receptor may also occur. The finding that fluoxetine did not influence the effect of quipazine, further confirms that quipazine acts by directly stimulating 5HT receptors.

The stimulatory action of quipazine on prolactin release was significantly reduced ($p<0.01$, unpaired t-test, 120 minute samples), and 5-hydroxytryptophan failed to stimulate prolactin secretion, in nest-deprived incubating birds (Fig. 24A). These results suggest that although 5HT stimulates prolactin secretion in the laying hen, its physiological importance in the release of prolactin during incubation may be small. Also, of the 5HT receptor blockers tested, only methysergide was effective in lowering the plasma levels of prolactin of incubating birds (Fig. 24B). The fact that methysergide can stimulate dopamine receptors as well as block 5HT receptors (Krulich et al. 1981) may explain this result. The reduction of plasma levels of prolactin in incubating hens by methysergide may reflect its ability to stimulate dopamine receptors in the pituitary gland, as dopamine has been shown to inhibit the stimulated release of prolactin from the isolated chicken (Harvey et al. 1982) and pigeon (Hall, 1982) pituitary glands.

The differences in responsiveness between laying, and nest-deprived-incubating birds to quipazine, 5-hydroxytryptophan, cyproheptadine and SQ 10631 suggests that hypothalamic 5HT is less important in the stimulation of prolactin release in broody than in laying birds. Further, it suggests that the extrapolation of theories of the neuroendocrine control of broodiness based solely on studies in immature birds or laying hens is not justified.

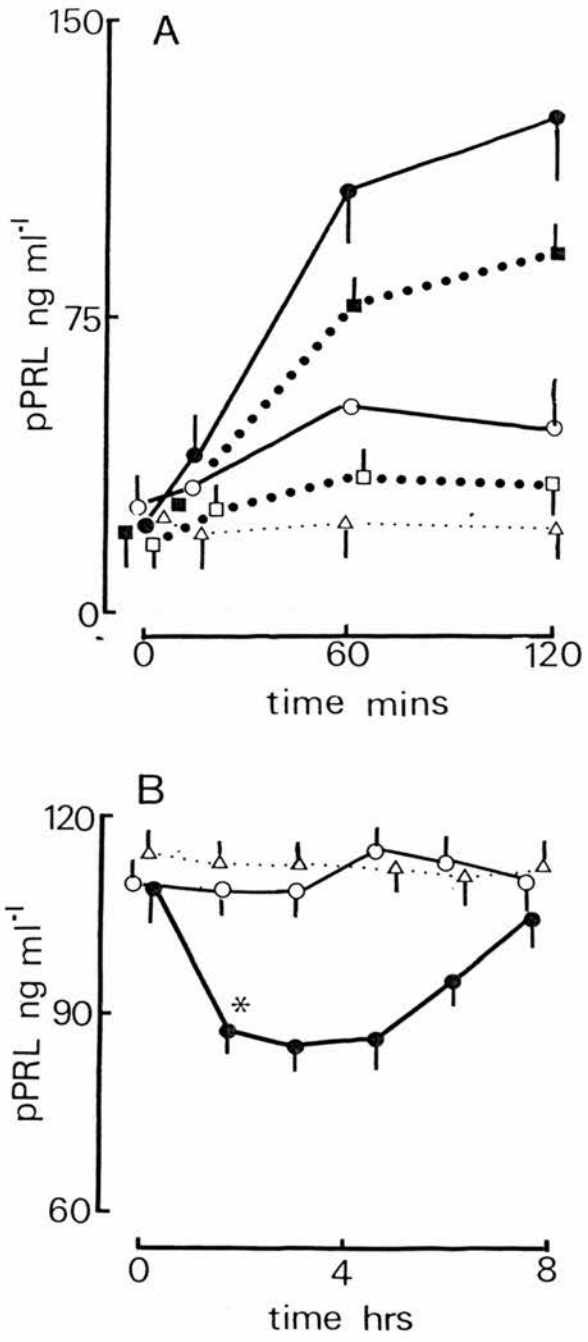


Figure 24: Pharmacological manipulations of plasma levels of prolactin. A: the effect of a single injection of 15 mg kg^{-1} quipazine (circles), 50mgkg^{-1} 5HTP (squares) or saline (triangles) on plasma levels of prolactin in groups (n=8) of laying (solid lines) or incubating bantam hens that had been deprived of their nests for 24 hours (broken lines). Results are the means ± SEM. B: The effect of a single injection of saline (Δ), 15mgkg^{-1} cyproheptadine (○) or 15mgkg^{-1} methysergide (●) on plasma levels of prolactin in groups (n=8) of incubating bantam hens. Results are the mean ± SEM, *$p < 0.05$ compared with preceding point, (paired t-test).

6.1.2.The effect of manipulation of 5HT activity on plasma levels of prolactin measured in the heterologous radioimmunoassay

When selected plasma samples were assayed for prolactin content in the heterologous radioimmunoassy, neither quipazine nor 5-hydroxytryptophan treatment elevated plasma levels of prolactin in laying bantam hens (Table 5). Conversely, the heterologous radioimmunoassay detects an acute elevation in plasma levels of prolactin after administration of porcine vasoactive intestinal polypeptide (See section 7), which the homologous assay does not. Figure 25 shows the result of measuring plasma samples from quipazine, 5-hyroxytryptophan and porcine VIP treated birds in both assay systems. Although the homologous and the heterologous radioimmunoassays are highly correlated when measuring the rise in plasma level of prolactin which occurs at the onset of incubation (Lea, 1982), no correlation exists after the drug treatments.

6.2 Effect of Quipazine on Prolactin Secretion In Vitro

The effect of quipazine on prolactin secretion was assessed in vitro by incubating the drug with pituitary glands and hypothalami from laying bantam hens either alone or together and measuring prolactin released into the incubation media. Prolactin was measured using both the homologous and heterologous radioimmunoassays to confirm their specificities.

Using the homologous radioimmunoassay, quipazine had no effect on the release of prolactin from isolated pituitary glands(Fig. 26A). Prolactin release was stimulated in pituitary/hypothalamus co-incubations and this release was enhanced in the presence of quipazine. The isolated hypothalamus released prolactin in the absence of quipazine, and this release was not enhanced after the addition of the drug (Fig 26B).

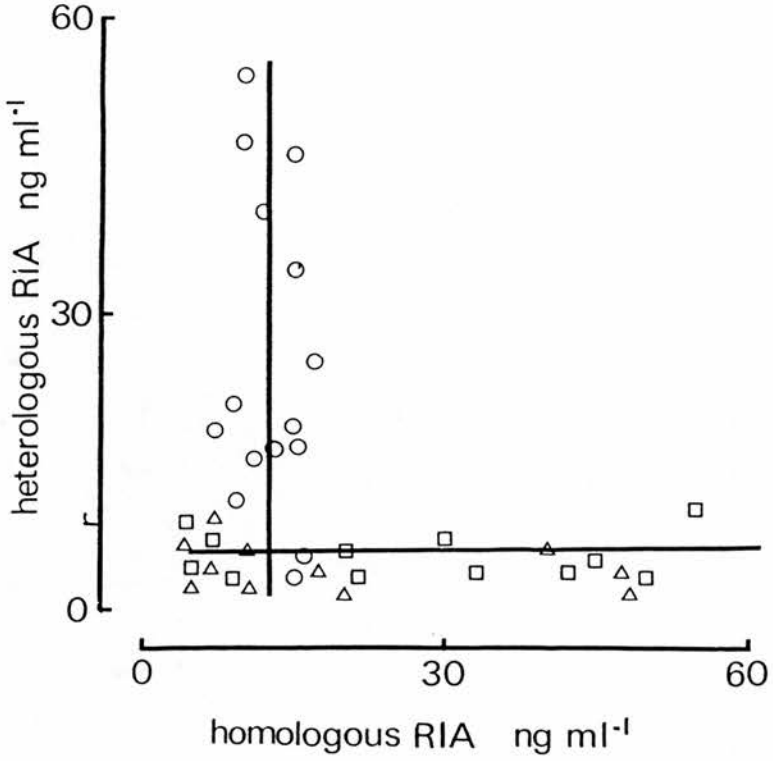


Figure 25: Comparison of homologous (Scanes et al 1976) and heterologous (McNeilly et al 1978) prolactin radioimmunoassays. Plasma taken samples from bantam hens immediately prior to, and at intervals up to 2 hours after, a single injection of quipazine ($15mgkg^{-1}$, squares), 5-hydroxytryptophan ($50mgkg^{-1}$, triangles) or vasoactive intestinal polypeptide ($100mgkg^{-1}$, circles) were measured for prolactin content in both assays. Lines of best fit calculated by linear regression ($p<0.01$, f-test) ran parallel with both axes.

Table 5: Comparison of the two prolactin assays. Plasma samples from groups (n=8) of laying bantam hens taken immediately before and 2 hours after a single injection of quipazine or 5-hydroxytryptophan (5HTP) were assayed for prolactin content in both the homologous and heterologous RIAs. The results are expressed as means ± SEM. Significant differences between mean prolactin levels at injection and 120 min later are marked by '*'. (*-$p<0.001$,paired t-test).

TREATMENT	PLASMA PROLACTIN($ngml^{-1}$) 0 mins	120	ASSAY
QUIPAZINE(15 $mgkg^{-1}$)	20.9±6.1	125.3±12.6*	homologous
	9.3±3.1	8.6±2.7	heterologous
5HTP (50 $mgkg^{-1}$)	23.4±5.2	87.3±15.6*	homologous
	8.2±2.5	10.2±4.1	heterologous

With the heterologous radioimmunoassay, quipazine had no effect on the release of prolactin from isolated pituitary glands. Prolactin release was not stimulated in pituitary/hypothalamus co-incubations in the absence or presence of quipazine (Fig 26C). Only relatively small amounts of immunoreactive prolactin were detected in incubations of hypothalami alone (Fig 26D).

These findings, together with those in the previous section, suggest that the homologous radioimmunoassay detects an immunologically active substance released from the hypothalamus ,the secretion of which may be stimulated by activation of 5HT receptors. This possibility was further investigated using polyacrylamide-gel electrophoresis, to separate components of immunoreactive material in the incubation media.

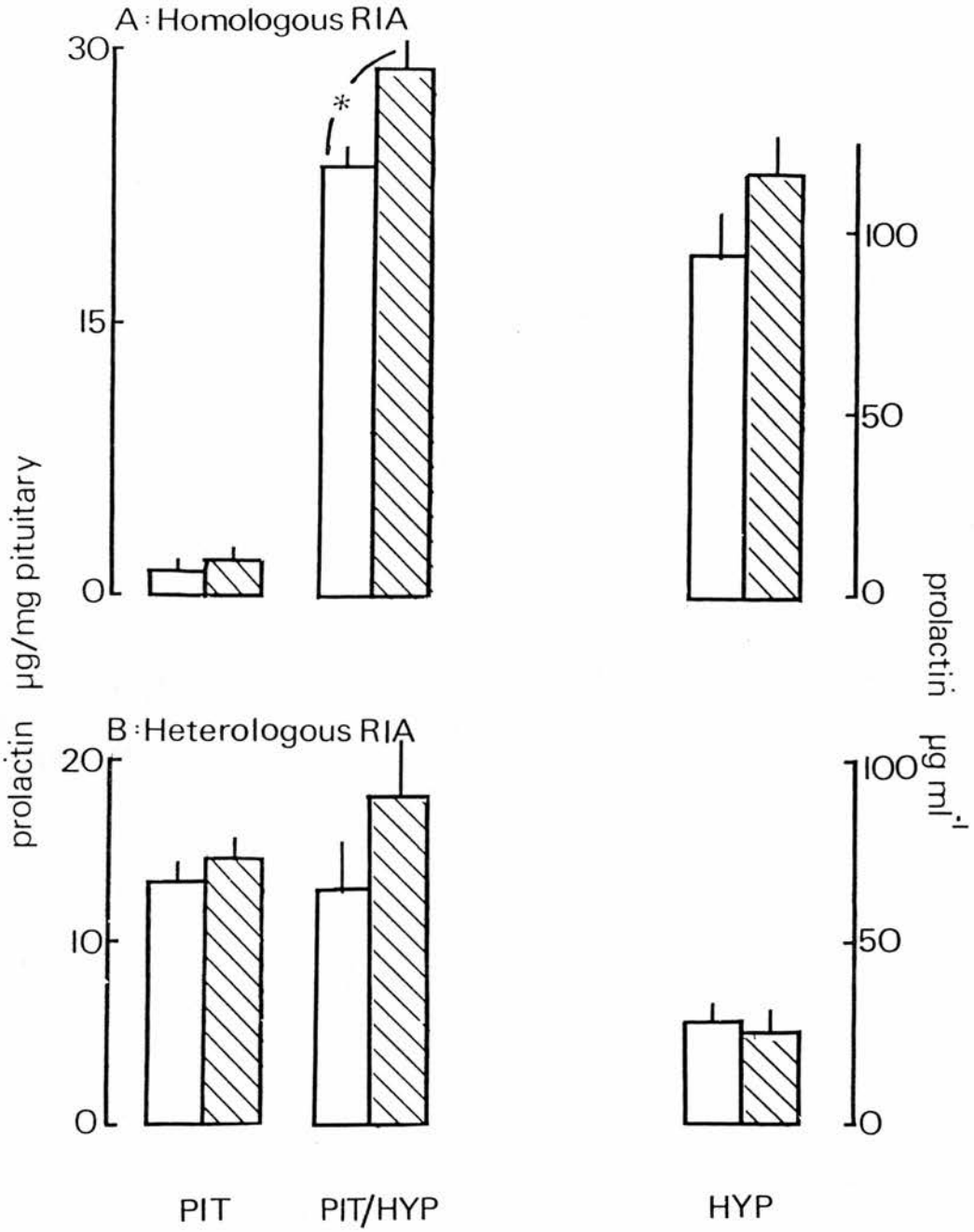


Figure 26: The effects of quipazine on prolactin secretion in vitro. The concentration of prolactin released during incubations of anterior pituitary glands (PIT), anterior pituitary glands and basal hypothalami (PIT/HYP), and hypothalami alone (HYP) in the presence (▨) and absence (□) of 10^{-7} M quipazine was measured in the homologous and heterologous radioimmunoassays. Results are the mean ± SEM of 6 independent observations (*$p<0.05$, unpaired t-test).

6.3 Polyacrylamide Gel Electrophoresis

Proteins in incubation media and aqueous extracts of anterior pituitary glands and neurohypophyses were separated by discontinuous polyacrylamide gel electrophoresis. Examples of the typical banding patterns seen after protein staining are shown in figure 27. One tract of two paired samples was stained to give the banding pattern while the other was cut into slices representing 0.05 Rf units and the proteins eluted from each finely homogenised slice. By assaying aliquots of the resulting eluent in the homologous radioimmunoassay 'prolactin' immunoprofiles were constructed. More than 90% of total immunoreactivity was found in the lower portions of the gels (Rf values 0.4). Peaks of immunoactivity correlated well with protein bands observed in the stained gel. A summary of the results in terms of the observed banding patterns and relative immunological activity associated with each band is given in figure 28.

An immunoactive protein band (Rf = 0.55) was present in all samples studied. The proportion of total immunoactivity concentrated in this band is higher in incubation media from isolated hypothalami and neural lobe extracts, than in anterior pituitary gland extract. An immunoactive protein band of Rf = 0.65 was also present in samples of neural lobe extract. Only samples from incubations containing pituitary glands from laying birds, co-incubations of pituitary glands from incubating birds with hypothalami and pituitary gland extract,contained a strongly immunoreactive protein band of Rf = 0.75. An immunoreactive protein band of Rf = 0.95 occurred only in samples containing pituitary glands from incubating birds.

In the system used, anterior pituitary gland extract contains 4 electrophoretically distinct proteins which make up its immunoreactivity in the homologous prolactin radioimmunoassay. Samples of medium from incubations containing isolated pituitary gland from laying birds contain two of these proteins (Rf = 0.55 and 0.75), suggesting that the proteins of Rf = 0.45 and 0.60 in the pituitary extract are not normally released by the gland. However, both neural

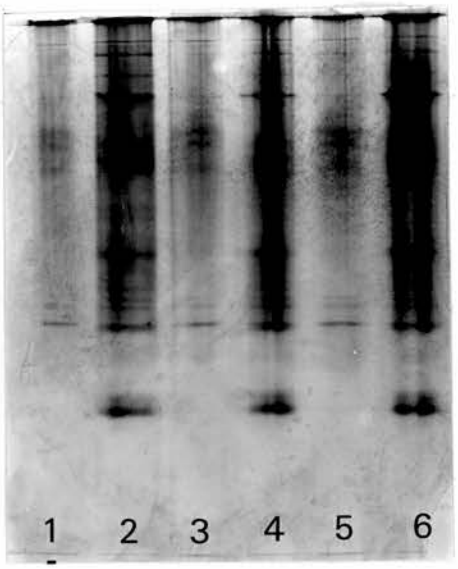


Figure 27: Photograph of stained gel. Anterior pituitary gland extract (even nos) and neural lobe extract (odd nos).

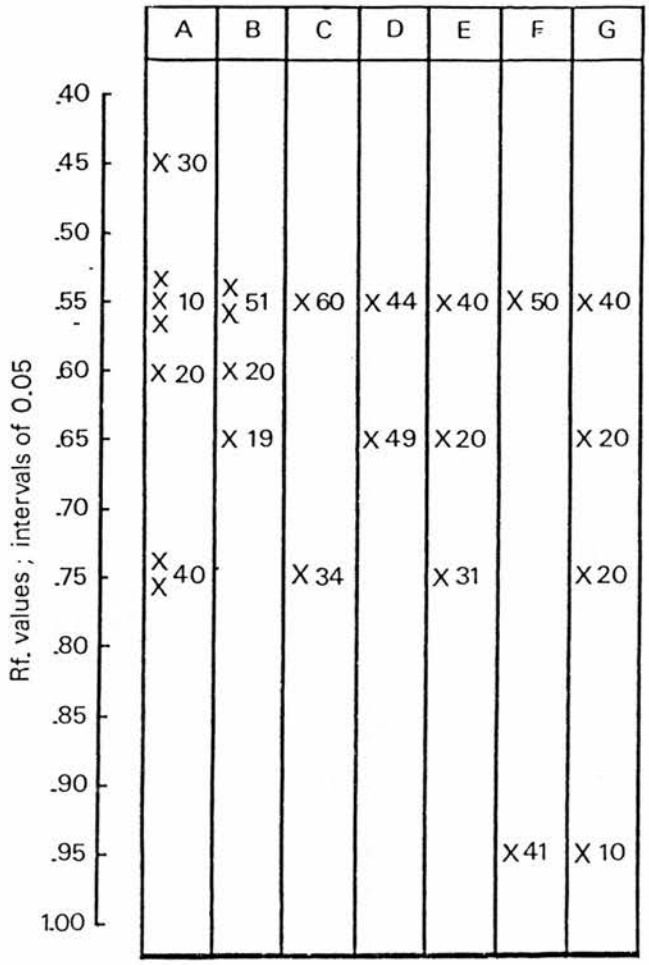


Figure 28: Banding patterns and immuno profiles after poly acrylamide gel electrophoresis of; A:anterior pituitary gland extract (n=4/2). B: neural lobe extract (n=4/2). C:incubation media of pituitary glands from laying hens (n=6/3). D:incubation media of basal hypothalami from laying hens (n=6/3). E:incubation media of co-incubation of pituitary glands and basal hypothalami from laying hens (n=6/2). F:incubation media of pituitary glands from incubating bantam hens (n=6/3). G: incubation media of co-incubations of pituitary glands and basal hypothalami from incubating hens (n=6/2).
The first number in parenthesis refers to the number of banding patterns studied while the second refers to the number of immunoprofiles constructed.

lobe extract and samples of media from incubations of isolated hypothalami also contain an immunoreactive protein of Rf = 0.55 which is electrophoretically indistinguishable from that seen in samples of pituitary gland incubations. An electrophoretically distinct immunoreactive protein (Rf = 0.65) released by the hypothalamus and which is present in neural lobe extract, does not occur in samples of pituitary gland incubations.

The differences between the proteins released by incubations of pituitary glands of laying and incubating birds, which make up the bulk of the immunoactivity in these samples, may reflect a difference in the molecular forms of releasable prolactin that are dependent upon physiological state. The presence of immunoreactive proteins of Rf = 0.95 in samples from coincubations of pituitary glands from incubating birds and hypothalami may also suggest a role for the hypothalamus in governing the type of prolactin secreted by the pituitary gland.

6.4 Conclusion: Implications for the Interpretation of Prolactin Radioimmunoassay Data

In a detailed study of the characteristics of the homologous prolactin radioimmunoassay, Lea (1982) found that immunoreactive 'prolactin' was present in extracts of hypothalamic tissue and present in large quantities in neural lobe extracts, as well as in the anterior pituitary gland. However, the amount of immunoreactive prolactin in hypothalamic extracts was less than the estimated total immunoactivity found in chicken plasma after intravenous injection of hypothalamic extract. The results of the present study confirm and extend these observations. Not only is immunoreactive prolactin present in the hypothalamus, it can be released in vitro and consists of two electrophoretically distinct components. Immunoreactive prolactin in anterior pituitary gland extract occurs as four major components, only two of which appear to be released in vitro. One of the components releasedby the pituitary was electrophoretically indistinguishable from one of the releasable hypothalamic proteins but neither are immunologically

distinct. Three immunologically active components are released by the pituitary and hypothalamus in co-incubation which reflects the sum of components released by the pituitary and hypothalamus when incubated alone.

On the basis of these findings, plasma prolactin levels in vivo and prolactin released during pituitary/hypothalamus co-incubations measured by the homologous radioimmunoassay, may reflect the sum of immunologically active proteins released by the hypothalamus and neural lobe, as well as prolactin released from the anterior pituitary gland.

The heterologous prolactin radioimmunoassay also detects prolactin-like immunoreactivity in extracts of chicken hypothalamus and neural lobe (Lea, 1982). However , this assay system detects only small amounts of immunoactivity released by the hypothalamus in incubation (Fig. 26D), which suggests that the immunoactivity it detects in hypothalamic extract is not released upon incubation of the hypothalamus. Thus the heterologous assay may be able to differentiate between prolactin released from the pituitary gland and substances released from the hypothalamus, that are immunoactive in the homologous system. A corollary of this possible difference between the two assay systems is the inability of the heterologous system to detect acute changes in plasma levels of prolactin after the administration of drugs which modify 5HT systems. These drug treatments are functionally non-specific since it is certain that not all 5HT neuronal systems are involved in the control of prolactin secretion and that the drugs used affect all 5HT systems. Further, because the homologous assay measures more than one immunoreactive substance it too can be considered as non-specific, as the balance of immunoreactive components released after drug administration may be altered dramatically.

However, the two assay system show strong positive correlations in situations where prolactin secretion is changed by an endogenous mechanism, as for example, during the transition between laying and incubating(Lea et al. 1981) and during nest deprivation(section 4.1.1).

Although 5HT may be involved in the physiological control of prolactin secretion in the chicken, its importance has perhaps been overestimated due to the assumption that immunologically active material released by drug action and detected by the homologous assay, is solely prolactin originating from the pituitary gland.

7.0 VASOACTIVE INTESTINAL POLYPEPTIDE AS A SPECIFIC PROLACTIN-RELEASING FACTOR IN THE BANTAM HEN

The aim of the following experiments was to determine in the bantam hen, whether neurones containing VIP-like immunoreactivity occur in the hypothalamus , and if so, to assess the effects of VIP on the release of prolactin in vivo and from the anterior pituitary gland in vitro.

7.1 The Distribution of VIP-Like Immunoreactivity in the Hypothalamus

The following section describes the immunohistochemical distribution of VIP-like material in the adult bantam hypothalamus. The distribution of neurones and terminals containing VIP-like immunoreactivity was plotted on the para-sagittal and transverse planes used by Sterling and Sharp (1981) in their study on the distribution of LHRH (Fig. 29).

7.1.1 The Mediobasal Hypothalamus

Small VIP-immunoreactive perikarya were present throughout the caudal portion of the infundibular nuclear complex and the lateral tuberal area (Fig. 29.F.). These cells were usually bi-polar, and the majority were orientated in a dorso-ventral plane. The immunoreactive reaction product was granular in appearance and was dispersed evenly throughout the cytoplasm and axonal processes of the cells. No reaction product was seen in the cell nuclei (see fig 30)

Heavily immunoreactive beaded fibres were present in the region of the perikarya. Fibres originating from the infundibular nuclear complex were seen to run laterally and ventrally, entering the median eminence by skirting the lateral horn of the infundibular recess and forming part of the tubero-hypophyseal tract described by Yamada and Mikami (1982). Small fibres and characteristically punctate VIP-immunoreactive terminals were abundant in the external layers of both the anterior and posterior median eminence. Fibres and terminals were observed in close apposition to capillary blood vessels in the anterior median eminence. Little immunoreactive material occurred in the internal layer of the median eminence (see figure 30)

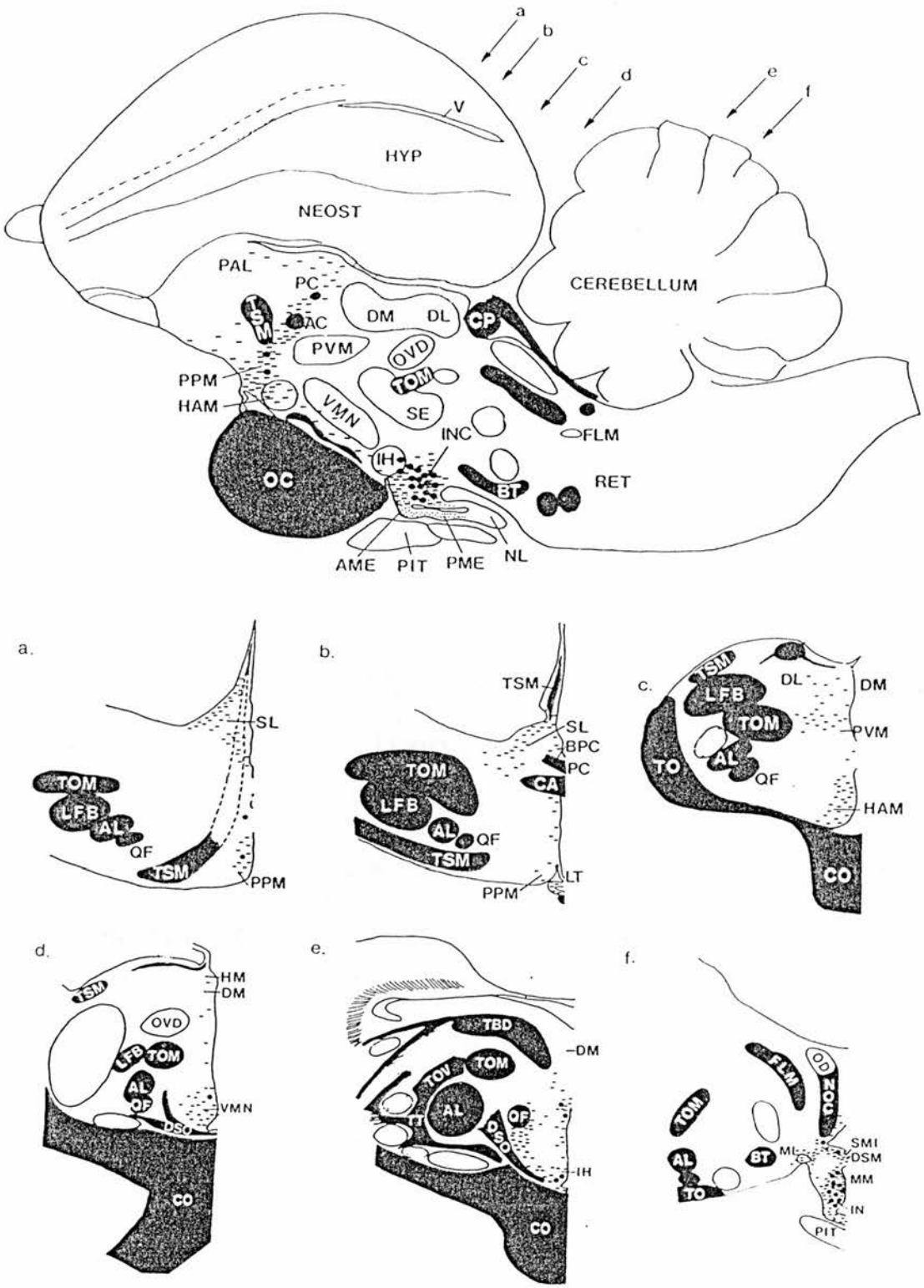
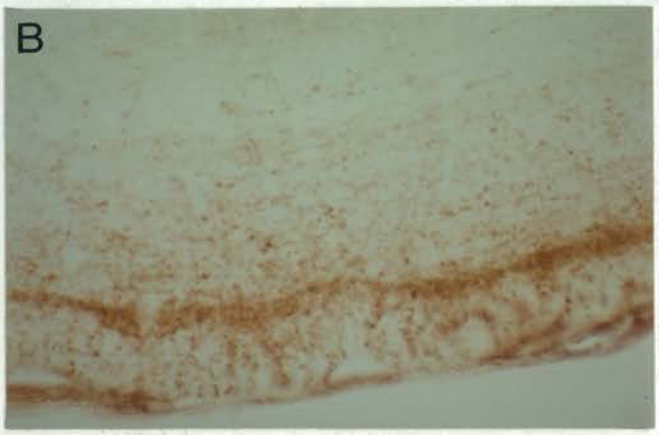


Figure 29: The Distribution of VIP-like immunoreactivity In The Bantam Brain. VIP was located immunohistochemically in para-sagittal sections of 5 preparations and transverse sections of 7 preparations and plotted on sagittal and transverse representation of the brain (Sterling and Sharp, 1982). Cells are represented by dots, fibres by dashes and terminals by stippling. For abbreviations see appendix.


A


B


C

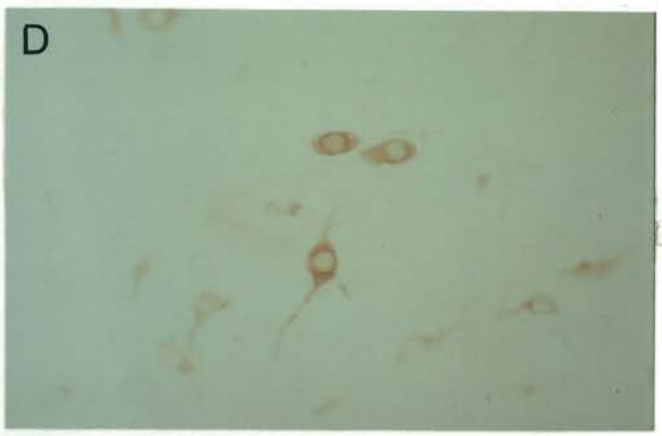


Figure 30: Photomicrographs of VIP-like immunoreactivity.

A. Transverse section through the mediam eminence (x10).
B. Sagittal section through the median eminence (x40).
C. Fibres in the pre-optic region (x20).
D. Cells in the infundibular neuclear complex (x10).
E. Cells in the infundibular neuclear complex (x40).

7.1.2 The Mid-Hypothalamus

A few VIP-immunoreactive perikarya were observed in the region lateral to the paraventricular organ (Figure 29.E,F). No liquor-contacting processes were observed but dense fibre staining occurred in this region. Fibres spread laterally from these cells to the area of the nucleus mammilaris lateralis and seemed to project forwards towards the anterior hypothalamus as well as descend towards the median eminence.

7.1.3 The Anterior Hypothalamus

A few small VIP-immunoreactive perikarya were observed in the medio-basal preoptic region (Fig 29.A). This area was rich in VIP-containing fibres and terminals which occurred in a broad band from above the pallial commissure and septal nuclei through the region of the anterior commissure to the lamina terminalis. The lamina terminalis contained an abundance of immunoreactive terminals (Fig. 29.B).Long beaded fibres running rostro-caudally between the pre-optic region and the tuberal area were observed in the area of the supra-optic tract and ventromedial nucleus(Fig. 29.B,C,D).

7.1.4 Discussion and Summary

It has been suggested that the tuberohypophyseal tract in birds consists of three distinct elements (Wingstrand 1951; Oksche and Farner, 1974). The VIP-immunoreactive neurones with cell bodies in the infundibular nuclear complex in both the bantam and quail hypothalamus (Yamada and Mikami, 1982) form tracts which correspond to the medial bundle of the tubero-hypophyseal tract, which passes along the margin of the third ventricle, and the lateral bundle, which passes along the ventral surface of the hypothalamus (Wingstrand 1951). Also, the VIP-immunoreactive perikarya close to the nucleus mammilaris lateralis send fibres to the median eminence along the posterior bundle of the tubero-hypophyseal tract (Oksche and Farner, 1974). All three divisions of the tubero-hypophyseal tract terminate in the external layer of the median eminence. The dense distribution of

VIP-immunoreactive fibres and terminals located in the bantam median eminence contrasts with the sparse distribution found in mouse and rat median eminence (Sims et al. 1980). The presence of VIP-immunoreactive terminals close to the portal capillaries in the median eminence suggests that VIP-like material is secreted into the portal vessels. Evidence of possible interaction between the VIP-like system and those of 5-hydroxytryptamine and catecholamine containing neurones, comes from the overlap in their anatomical distribution in the hypothalamus and median eminence(see section 3). The distribution of VIP-like immunoreactive neurones also coincides with the distribution of LHRH perikarya and terminals(Sterling and Sharp 1981), and with fibres containing somatostatin (Blasher ,1983) and neurotensin (Yamada and Mikami, 1981). In summary, neurones containing VIP-like immunoreactivity occur in the bantam hypothalamus with a distribution consistent with a role in the regulation of hormone secretion from the anterior pituitary gland. This regulatory role may involve a direct effect of VIP released into the portal vessels acting directly on the pituitary gland and/or interaction of VIP fibres with other peptidergic or monoaminergic systems in the median eminence and the hypothalamus.

7.2 The Effect of VIP on the Pituitary Gland <u>in vitro</u>

The effect of porcine VIP on prolactin release from the isolated pituitary gland is demonstrated in Figure 31. The presence of 10^{-7} M porcine VIP in the incubation medium had no effect on prolactin secretion from pituitary glands from laying hens, but significantly stimulated ($p<0.001$, unpaired t-test) prolactin release from pituitary glands from incubating hens.

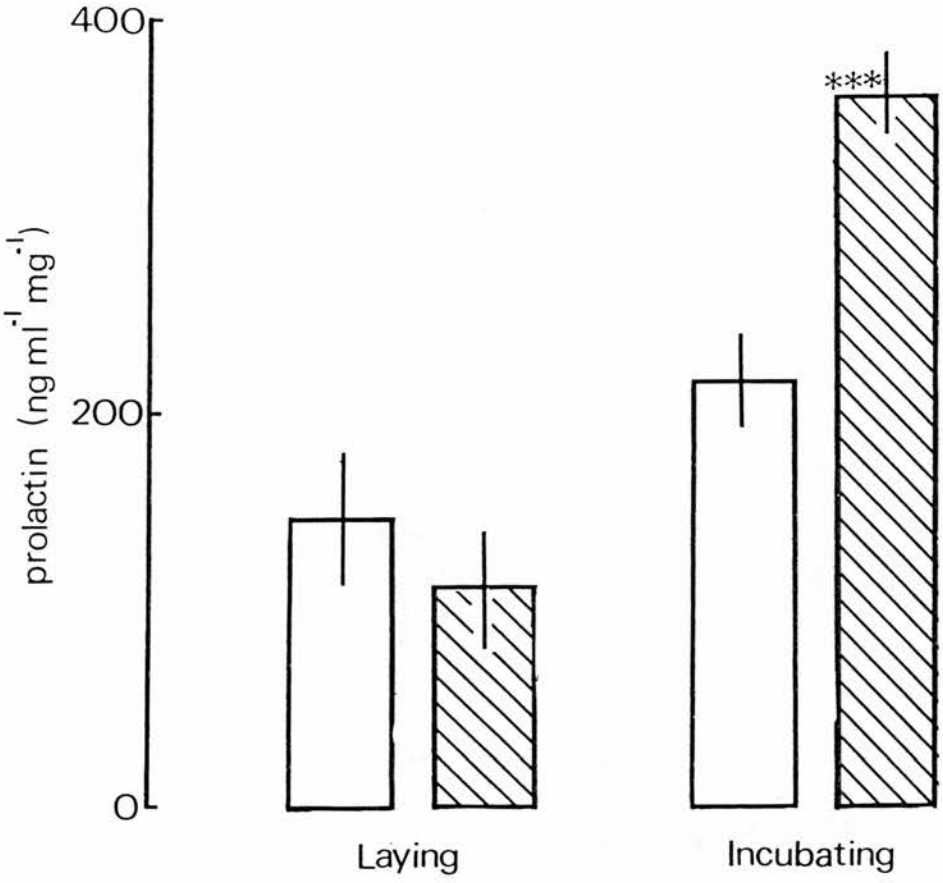


Figure 31: Effect of porcine VIP on prolactin secretion from pituitary glands incubated in vitro. Pituitary glands for laying and incubating bantam hens were incubated in the presence (▨) or absence (□) of 10^{-7} M VIP. The prolactin content of the medium was assessed in the heterologous radioimmunoassay. The results are expressed as the mean ± SEM of eight observations, (** = $p<0.001$, unpaired t-test).

7.3 The Effect of VIP on Hormone Secretion In Vivo

Plasma samples from a series of experiments in which porcine VIP was administered to laying, nest-deprived-incubating (NDI) and ovariectomised bantams were assayed for prolactin content by the heterologous radioimmunoassay, for LH content by a homologous radioimmunoassay and for growth hormone content by a homologous radioimmunoassay.

7.3.1 Time Course

Plasma levels of prolactin , LH and GH were assessed in NDI bantam hens after administration of 100 μg kg^{-1} porcine VIP (Figure 32). Two minutes following injection plasma levels of prolactin were significantly elevated compared with their pre-injection values ($p \leq 0.01$, paired t-test) and those of saline injected birds ($p < 0.01$, unpaired t-test). Plasma levels of prolactin in VIP treated birds continued to rise to a peak ten minutes after injection, which was 14 fold higher than control values. A gradual decline occurred over the following twenty minutes, bringing prolactin levels in VIP treated birds close to those of control birds 30 minutes after saline injection. The plasma levels of LH in VIP treated birds were also significantly increased above those in control birds two minutes after injection ($p < 0.05$, unpaired t-test). The stimulation of LH secretion, although equally acute in onset, was of a lesser magnitude and less sustained than the stimulation of prolactin secretion. Ten minutes after injection, plasma levels of LH in VIP treated and control birds were statistically indistinguishable. Plasma levels of GH showed large individual variations and when grouped together plasma levels of GH in VIP treated birds did not significantly differ from those found in control birds at any time after injection.

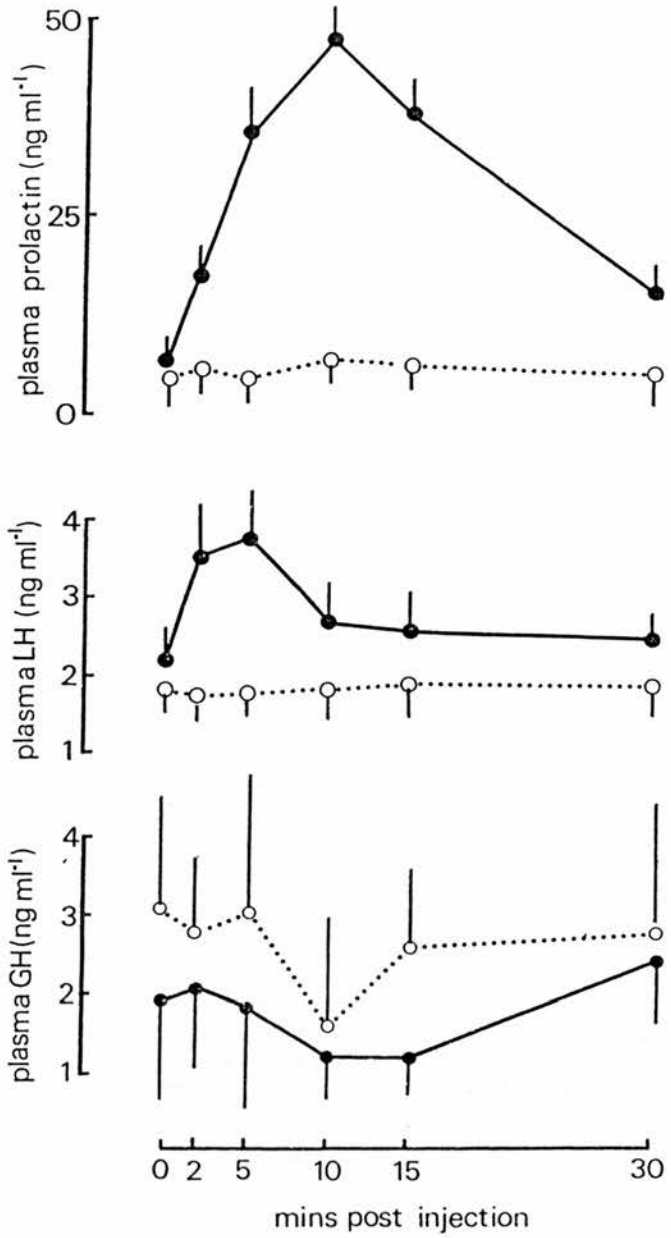


Figure 32: Effect of VIP on plasma levels of prolactin, luteinizing hormone (LH) and growth hormone (GH) in vivo. Groups of incubating bantam hens (n=5) which had been deprived of their nests for 24 hours were injected intravenously with either 0.75% saline (○) or 100 µg kg^{-1} porcine VIP (●). Blood samples were taken immediately before and 2, 5, 10, 15 and 30 minutes after injection. Plasma samples were assayed for prolactin content in the heterologous RIA and for LH and GH in the homologous RIA's. The results are expressed as means ± SEM.

7.3.2 Dose-Response Relationship

The effect on plasma levels of prolactin, LH and GH of various doses of VIP was determined in NDI bantam hens. All doses tested above and including 12.5 $\mu g\ kg^{-1}$ of VIP, significantly elevated plasma levels of prolactin 10 minutes after injection when compared with those 10 minutes after a control injection of saline (Figure 33). No increase in plasma levels of LH or GH were observed 10 minutes after VIP injection. Plasma levels of LH and GH in birds before and 10 minutes after injection of 50 and 100 $\mu g\ kg^{-1}$ are given below;

Table 6: The effect of VIP on plasma levels of LH and GH.

Dose of VIP ($\mu g\ kg^{-1}$)	**Plasma LH** ($ng\ ml^{-1}$)		**Plasma GH** ($ng\ ml^{-1}$)	
	0	10	0	10
0	2.10 ± 0.40	2.04 ± .032	1.6 ± 0.7	1.7 ± 0.5
50	2.24 ± 0.35	2.19 ± 0.30	1.3 ± 0.4	1.2 ± 0.3
100	1.80 ± 0.40	2.71 ± 0.50	1.8 ± 0.6	1.9 ± 0.7

Figures are for plasma luteinizing hormone (LH) and growth hormone (GH), before (0) and 10 minutes after (10) injections of VIP, and are the mean of 5 observations ∓ S.E.M.

7.3.3 The Effect of VIP on Plasma Levels of Prolactin in laying, Nest-Deprived-Incubating and Ovariectomised Bantam Hens

Groups of laying, nest-deprived-incubating (NDI) and ovariectomised birds were injected with 50 $\mu g\ kg^{-1}$ porcine VIP. Injections of VIP significantly elevated plasma levels of prolactin in laying and NDI but not in ovariectomised birds (Figure 34). The magnitude of the rise in plasma levels of prolactin was significantly greater in NDI than laying birds ($p<0.01$, unpaired t-test). Plasma levels of LH and GH were unaffected by VIP treatment.

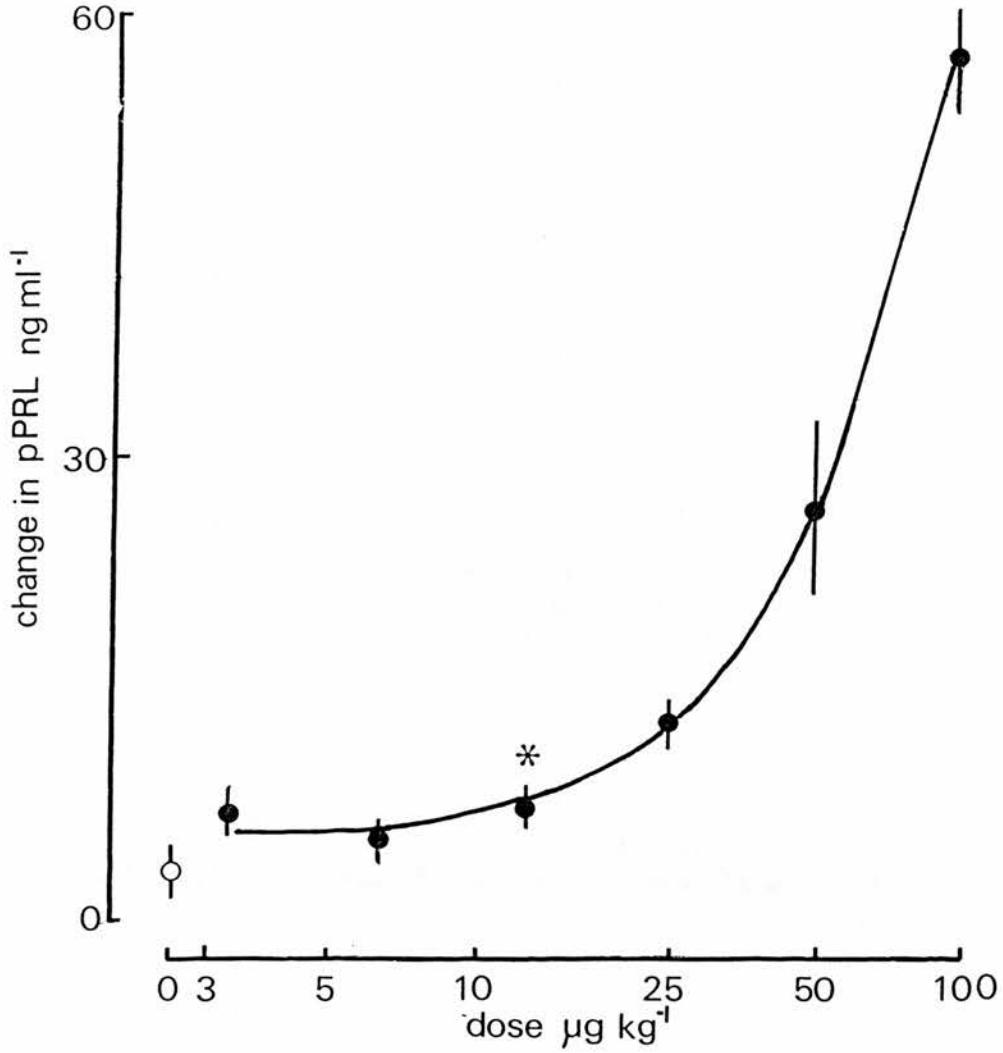


Figure 33: Dose response relationship. Groups of incubating bantam hens which had been deprived of their nests for 24 hours were injected intravenously with either 0.7% saline (O) or various doses of porcine VIP (3.25 - 100 µg kg^{-1}). Blood samples taken immediately prior to and 10 minutes after injection, were assayed for prolactin in the heterologous RIA. The results are expressed as the incremental rise in plasma level of prolactin and are the mean ± SEM's of 5 observations. The lowest dose of porcine VIP that significantly stimulated prolactin secretion ($p<0.05$, unpaired t-test), 12.5 µg kg^{-1}, is marked with an asterisk.

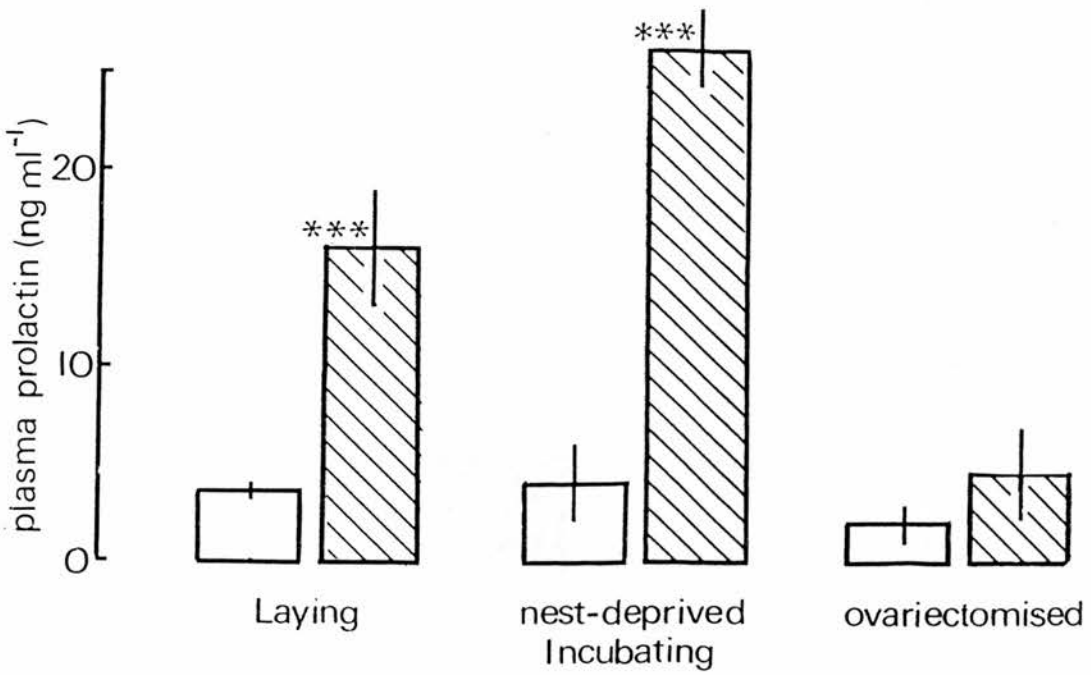


Figure 34: The effect of porcine VIP on plasma prolactin in laying, nest-deprived incubating and ovariectomised bantam hens. Groups of bantam hens (n=5) were injected intravenously with either 0.75% saline (□) or 50 µg kg^{-1} porcine VIP (▨). Blood samples were taken 10 minutes after injection and the plasma assayed for prolactin content in the heterologous RIA. The results are expressed as the mean ± SEM, (*** $p<0.001$, unpaired t-test).

7.4 Summary and Conclusion; VIP as a specific prolactin releasing factor

1. Neurones containing a VIP-like substance were demonstrated in the bantam hypothalamus. These neurones were distributed in such a way as to suggest their participation in the control of hormone secretion from the anterior pituitary gland.
2. Porcine VIP stimulated prolactin secretion from isolated pituitary glands from incubating birds but did not influence prolactin secretion from pituitary glands from laying hens.
3. Porcine VIP in low doses rapidly elevated plasma levels of prolactin when administered to nest-deprived-incubating (NDI) bantam hens.
4. Administration of porcine VIP resulted in the elevation of plasma levels of prolactin in NDI and laying, but not in ovariectomised bantam hens.
5. The administration of porcine VIP did not influence plasma levels of LH,except at the highest dose in NDI birds.
6. The administration of porcine VIP to laying, NDI or ovariectomised hens did not influence plasma levels of growth hormone.

On the basis of these results it would appear that VIP is a specific prolactin releasing factor of possible physiological significance in the bantam hen. The inability of porcine VIP to stimulate prolactin release from isolated pituitary glands of laying hens suggests that the prolactin releasing activity of porcine VIP when administered in vivo to the laying hen may result from its action at a non-pituitary, possibly hypothalamic, site. Alternatively, the differential effect of porcine VIP on pituitary glands from laying and incubating birds in vitro, and on plasma levels of prolactin in laying and incubating birds in vivo, may be due to a change in pituitary responsiveness to VIP ,which may be responsible for the maintenance of high plasma levels of prolactin in incubating birds. The mechanism of this change in responsiveness may be related to the change in ovarian steroid levels at the onset of incubation or long-term exposure to steroids during laying. The

facilitatory effect of ovarian steroids on the prolactin releasing activity of VIP is suggested by the inability of porcine VIP to stimulate prolactin secretion in ovariectomised birds.

8.0 NEUROENDOCRINE FACTORS INFLUENCING BROODINESS IN THE BANTAM HEN

This section summarises the principal findings in this thesis and integrates them into a model of the neuroendocrine control of broodiness in the bantam hen. It is acknowledged that hypothalamic neurotransmitter/neuropeptide systems other than those examined may also be involved in the expression of broodiness.

Figure 36 is a schematic diagram of possible neuroendocrine interactions involved in the control of broodiness. The diagram is composed of several major components:- a)'Hypothalamic drive', which results from the integration of internal stimuli (via the 'higher centres') and external (environmental) stimuli, and governs the overall activity of the hypothalamic neuroendocrine system, b) the LHRH system, which has been described in previous sections and is directly responsible for the control of LH secretion, c) the prolactin-releasing-factor (PRF) system which is directly concerned with the stimulation of prolactin secretion, d) the anterior pituitary gland which contains the LH and prolactin secreting cells, and e) the ovary which is the target of gonadotrophic activity and is the source of steroid hormones.

The transition between laying and incubation is triggered by a change in 'hypothalamic drive'. This change results from the action of environmental stimuli such as the photoperiod, the availability of a nest site and the presence of a clutch of eggs, coupled with endogenous stimuli resulting from a sustained period of egg production. The effect of such a change in hypothalamic drive is to alter the hen's physiology, through changes in the endocrine system, to permit incubation. If incubation is sucessful the hatched young contribute further environmental stimuli to promote brooding behaviour.

The object of this study was to elucidate the role of the hypothalamic monoamine neurotransmitters in transforming 'hypothalamic drive' into changes in the secretion of prolactin and LH. The role of the monoamine neurotransmitters in the control of broodiness by the regulation of the release of LHRH and PRF are considered first. Secondly the possible identity of PRF is considered.

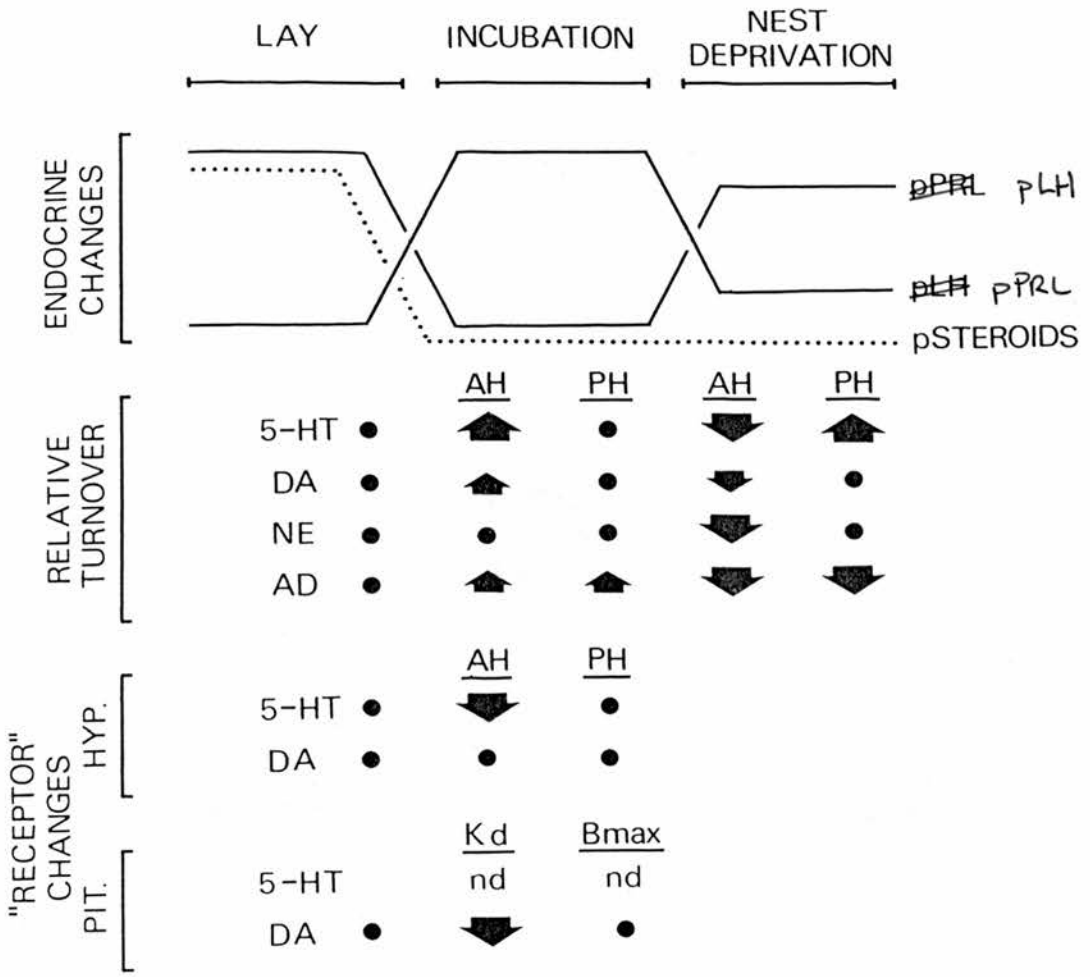


Figure 35: Summary of findings. The upper part of the figure is a schematic representation of endocrine changes investigated. The magnitude and direction of changes in monoamine turnover and putative receptor populations, relative to those in the preceding endocrine state are indicated by arrows.

AH = anterior hypothalamus ; PH = posterior hypothalamus .

Kd = apparent dissociation binding constant , Bmax = maximum number of binding sites in the tissue.

8.1 THE ROLE OF HYPOTHALAMIC MONOAMINE NEUROTRANSMITTERS AND THEIR RECEPTORS IN BROODINESS

The relative changes in the turnover of hypothalamic monoamines, and changes in putative 5-hydroxytryptamine and dopamine receptors in the hypothalamus and pituitary gland, which correlate with the endocrine changes of broodiness are summarised in figure 35. In integrating these findings into figure 36 it was assumed;

a) that with few exceptions the changes in monoamine functional activity observed are related to the changes in LH and/or prolactin secretion.

b) that the quantal relationship between receptor activation and intracellular response,was the same in laying and incubating birds.

After the transition from laying to incubating eggs the relative increase in the turnover of 5HT in the hypothalamus is positively correlated with plasma levels of prolactin and inversely correlated with plasma levels of LH. The same relationship between 5HT turnover in the anterior hypothalamus and plasma prolactin and LH occurs in incubating birds when they are deprived of their nests. The relative increase in 5HT turnover in the anterior hypothalamus between laying and incubating birds may be due to the changes in putative 5HT receptor populations of the anterior hypothalamus. In mammals a 5HT autoreceptor has been described, which when stimulated inhibits the activity of the 5HT neurone (Cerrito and Raiteri, 1979; Baumann and Waldmeier, 1981; Aghajanian, 1982; Martin and Saunders-Bush, 1982). Evidence presented earlier (section 5) suggests that a novel population of putative 5HT receptors, located on 5HT neurones, occurs only in the anterior hypothalamus of laying birds. If these putative receptors are inhibitory to the function of the neurones on which they are located then their disappearance would permit an increase in turnover similar to

that in the incubating birds. A possible mechanism for this change in putative receptor populations comes from studies in ovariectomised rats, where it has been demonstrated that the level of 5HT receptors in the anterior hypothalamus is increased by oestrogen treatment (McEwan and Parsons, 1982). The onset of incubation in the bantam hen is accompanied by ovarian regression and a pronounced decrease in the plasma levels of ovarian steroids (Sharp et al. 1975). If the unique putative 5HT receptor population in the laying anterior hypothalamus was also oestrogen dependent then this drop in steroid levels could lead to a drop in receptor levels. Thus in Figure 36, in the anterior hypothalamus of laying birds 5HT turnover is kept under a short-loop negative feedback which is reduced at the onset of incubation.

Since the ovary is regressed in incubating bantams, it is unlikely that the increase in plasma LH observed after short-term nest deprivation would stimulate steroid production sufficiently to promote the re-formation of putative 5HT autoreceptors. An alternative explanation must therefore be found to account for the rapid decrease in 5HT turnover in the anterior hypothalamus seen after nest-deprivation. Possibly this is related to the dramatic change in sensory input to the hypothalamus, mediated by unidentified higher control centres, which alters 'hypothalamic drive'.

It is possible that changes in 5HT turnover in the anterior hypothalamus at the onset of incubation may be associated with the inhibition of LH secretion rather than with the stimulation of prolactin secretion. Anatomically this suggestion is consistent with the presence of LHRH-immunoreactive cells in this area (Sterling and Sharp, 1982) and pharmacologically, with the effects on gonadotrophin secretion or gonadal function, of drugs which alter 5HT function. For example, treatment with the 5HT neurotoxin, 5,7-dihydroxytryptamine, augments photoperiodically induced testis growth in chickens (Calas, 1975), while drug treatments which inhibit 5HT synthesis augment, and drugs which elevate 5HT synthesis inhibit, photostimulated testicular growth in quail (El Halawani et al. 1978). Further, in the incubating turkey, drug treatments which reduce 5HT synthesis result in a rise in plasma levels

of LH (EL Halawani et al. 1983) while pharmacological inhibition of 5HT synthesis or the administration of the 5HT antagonist methysergide, depresses plasma levels of LH in cockerels (Buonomo et al. 1981). These observations are consistent with an inhibitory action of 5HT neurones on the activity of neurosecretory cells containing LHRH. Thus in Figure 36 an inhibitory pathway is shown between hypothalamic 5HT and LHRH systems which is more prominent in the incubating than in the laying bird.

Although the turnover of 5HT and putative 5HT populations do not change after the initiation of incubation, a rapid increase in 5HT turnover occurs after nest-deprivation. This increase may reflect changes in the activity of 5HT neurones in the posterior hypothalamus which are unrelated to the control of prolactin and LH secretion. One possibility is that it is related to a stress-induced release of ACTH. In Figure 36 the possibility of 5HT stimulation of PRF release is acknowledged by the weak stimulatory pathway 5HT- and PRF-containing neurones.

In the anterior hypothalamus the relative turnover of dopamine is also inversely correlated to LH secretion and positively correlated to prolactin secretion. The possibility that dopaminergic neurones in this area are primarily concerned with the inhibition of LH secretion, is suggested by the decrease in hypothalamic dopamine turnover during ovarian regression in the turkey (El Halawani and Burke, 1976) and by the inhibitory effects of dopamine on photoperiodically induced testis growth in the quail (El Halawani et al. 1978). However dopaminergic neurones in the posterior hypothalamus may be involved in the inhibition of prolactin secretion. Although the turnover of dopamine in the posterior hypothalamus does not change after the onset of incubation, there is a decrease in the density of putative dopamine receptors in the pituitary gland. This change may permit the sustained secretion of the prolactin seen in the incubating bird by decreasing the responsiveness of the pituitary gland to the inhibitory effect of dopamine (Harvey et al. 1982). It is possible that the decrease in putative dopamine receptors in the pituitary may occur in response to declining levels of ovarian steroids at the onset of incubation. This effect of oestrogen on dopamine receptors is suggested by studies on the mammalian brain and

pituitary gland (McEwan and Parsons, 1982). Alternatively, the apparent decrease in putative dopamine receptors may be due to the hypertrophy of prolactin secreting cells in the incubating bird resulting in a larger cell membrane, with no alteration in absolute receptor concentration. On the basis of these findings, an inhibitory pathway is suggested between hypothalamic dopaminergic neurones and the LHRH system, and between dopamine and prolactin containing cells in the pituitary, in Figure 36. An inhibitory influence of dopamine on pituitary prolactin cells may be diminished by down-regulation of dopamine receptors in the pituitary of the incubating bird.

The turnover of hypothalamic noradrenaline did not change after the onset of incubation. Although noradrenaline is thought to exert a stimulatory control over LH secretion in young chickens (Buonomo and Scanes, 1983), the present results suggest that it is not involved in the regulation of the endocrine events of broodiness. The significance of the paradoxical drop in the turnover of noradrenaline in the anterior hypothalamus after nest deprivation requires further investigation and may be related to changed sensory input to the hypothalamus. For this reason, a strong stimulatory pathway is suggested between noradrenergic neurones and the LHRH system in the hypothalamus of the laying bird. This stimulatory pathway appears to be less important in the incubating bird.

The turnover of adrenaline in both parts of the hypothalamus was inversely correlated with LH secretion and positively correlated with prolactin secretion. This relationship is particularly noticeable when comparing incubating and nest-deprived incubating birds. Although little studied in relation to prolactin secretion and broodiness, the activity of adrenaline containing neurones may be the basis of the reciprocal relationship between LH and prolactin secretion. Thus, it is possible that adrenergic neurones in the anterior hypothalamus are inhibitory to LH secretion while adrenergic neurones in the posterior hypothalamus stimulate prolactin secretion. For this reason an

inhibitory pathway is suggested between adrenergic neurones and the LHRH system and a stimulatory pathway between adrenergic neurones and the PRF system (Figure 36).

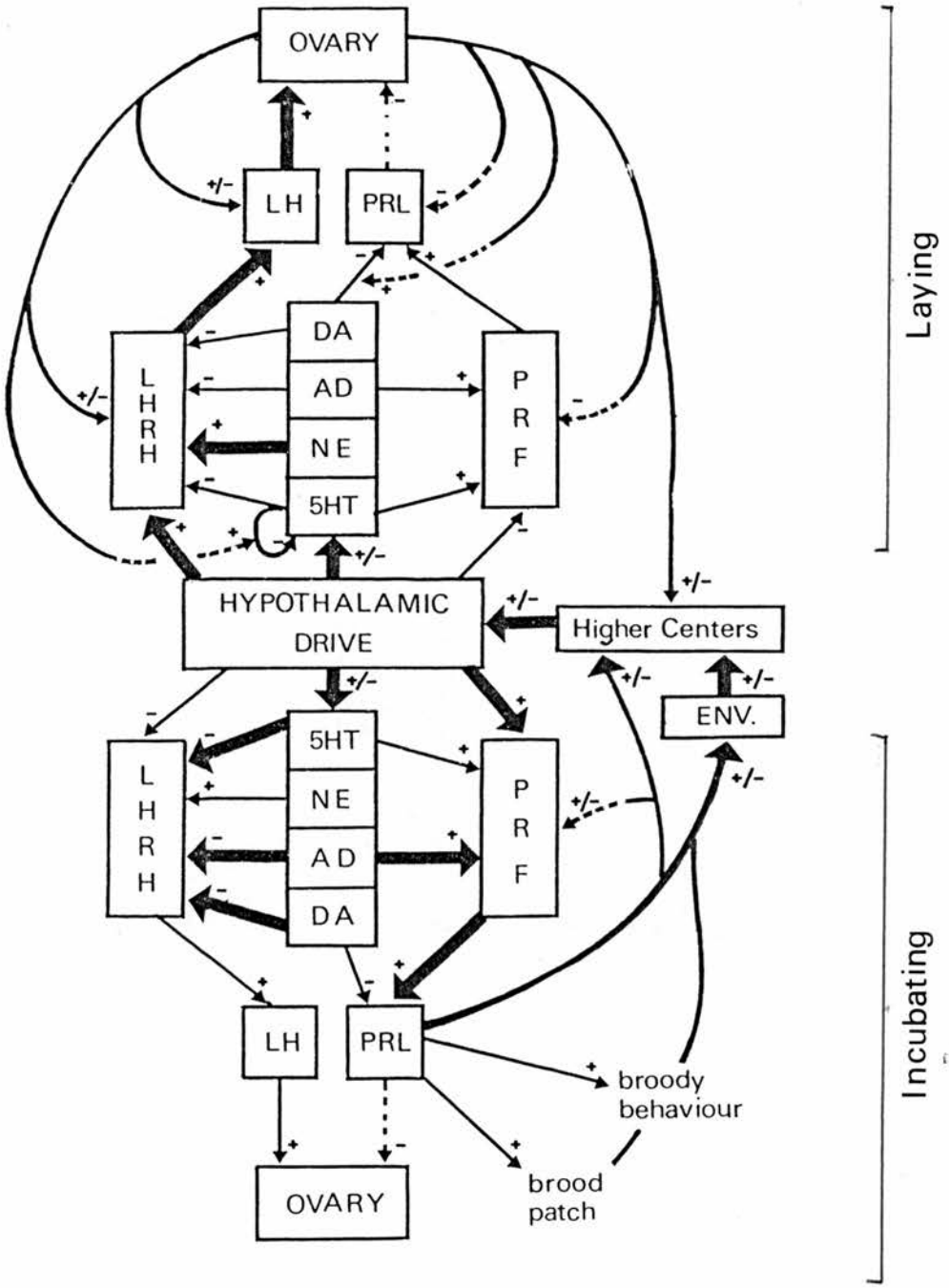


Figure 36: Neuroendocrine interactions in the control of broodiness. The magnitude of an interaction is determined by size of arrow. Broken lines represent putative interactions. Full explanation of the figure is made in the text (p.141 et seq.)

8.2 IDENTIFICATION OF A PHYSIOLOGICAL PROLACTIN RELEASING FACTOR:- VIP AS A POSSIBLE CANDIDATE

In the bantam hypothalamus a VIP-like substance was located in neurones which had an anatomical distribution consistent with a role in the control of anterior pituitary gland function. In support of this view porcine VIP selectively stimulated prolactin secretion from the pituitary gland in vitro. Pituitary glands from incubating birds were more responsive to porcine VIP than those from laying birds. The prolactin-releasing potency of porcine VIP in vivo was also dependent on other aspects of physiological state. Prolactin secretion was not stimulated by porcine VIP in ovariectomised hens but was stimulated in laying hens, and to a greater extent in incubating hens which were deprived of their nests. These findings suggest that the sensitivity of the pituitary gland to porcine VIP is dependent both on the releasable pool of prolactin and on the priming effect of ovarian steroids. Chronic exposure to high levels of ovarian steroids during a prolonged period of laying may trigger the initial rise in prolactin secretion by modifying the hypothalamic drive, or more specifically by altering the balance of prolactin inhibiting and releasing factor activity. The initial rise in prolactin secretion provokes broody behaviour, which in turn leads to an increase in the releasable pool of prolactin in the pituitary gland. While not excluding the possibility of a positive short-loop feedback of prolactin on VIP neurones in the hypothalamus, an increase in the releasable pool of prolactin probably results in an increased pituitary sensitivity to the prolactin-releasing activity of endogenous VIP-like material. Alternatively, the increase in the turnover of adrenaline in the posterior hypothalamus after the onset of incubation may stimulate the release of VIP-like material and thereby stimulate prolactin secretion. Although further investigation is required, the present series of experiments suggest that a VIP-like substance is a specific physiological prolactin releasing factor in the bantam hen. In support of this view, porcine VIP did not significantly stimulate the secretion of either growth hormone or luteinizing hormone. This proposition does not exclude the undoubted existence of other

prolactin-releasing factors, but suggests that comparisons should be made between other PRF candidates and VIP to assess further their physiological significance.

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10.0: ANATOMICAL ABBREVIATIONS

AC anterior commissure,
AL ansa lenticularis,
AM nucl. anterior medialis hypothalami,
AME anterior median eminence,
BC brachium conjunctivum, magnocellularis,
BPC bed nucleus of pallial commissure,
BT tr. bulbo-thalamicus,
CO optic chiasma,
CP posterior commisure,
DA tr. dorso-archistriaticus,
DL nucl. dorsolateralis,
DM nucl. dorsomedialis,
DSM decussatio supramamillaris,
DSO decussatio supraoptica dorsalis and ventralis,
FLM fasciculus longitudinalis medialis,
HAM nucl. hypothalamicus anterior medialis,
HM nucl. habenularis medialis,
HYP hyperstriatum,
IC nucl. intercalatus,
IH nucl. inferior hypothalami,
IN nucl. infundibuli,
INC infundibular nuclear complex,
LC nucl. linearis caudalis,
LFB lateral forebrain bundle,
LHy nucl. hypothalamicus lateralis,
LT lamina terminalis,
ML nucl. mamillaris lateralis,
MM nucl. mamillaris medialis,
NEOST neostriatum,
NL neural lobe,
NOC nervus oculomotorius,
OC optic chiasma,
OD nucl. oculomotorius dorsomedialis,
OVD nucl. ovoidalis,
PAL paleostriatum,
PC pallial commissure,
PHN nucl. hypothalami posterioris,
PIT pituitary,
PME posterior median eminence,
POM nucl. praeopticus medialis,
POR pre-optic region,
PPM nucl praeopticus paraventricularis
PVM nucl. paraventricularis,
PVO paraventricular organ,
QF tr. quinto-frontalis,
R Raphe
RET nucl. reticularis superior,
RU nucl. ruber,
SCT tr. striatrotegmentalis et stricocerebellaris,
SE stratum cellulare externum,
SM nucl. supramamillaris interstitialis,
SMD tr. septomesencephalicus (dorsalis),
SL nucl. septalis lateralis,
TBD tr. tecto-bulbaris dorsalis,
TO tr. opticus,
TOM tr. occipitomesencephalicus,
TOv tr. nuclei ovoidalis,
TRO nervus trochlearis,
TS tr. tectospinalis,
TSM tr. septo-mesencephalicus,
TT tr. tecto-thalamicus,
VM nucl. ventromedialis hypothalami